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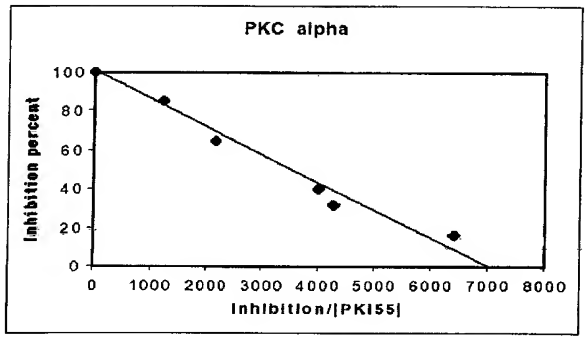
(54) Title: PROTEIN KINASE C MODULATORS, THEIR AMINOACID AND NUCLEOTIDE SEQUENCES AND USES THEREOF

(57) Abstract: The present invention provides novel polynucleotides and proteins encoded by such polynucleotides capable of modulating the Protein Kinase C activity for therapeutic, diagnostic and research purposes. The polypeptides according to the invention bind Protein Kinase C when this is in an activated form. According to preferred embodiments such polypeptides correspond to SEQ ID NO 2, 4, 5, 7, 9, 11, 12, 13. The invention also provides for the nucleotidic sequence encoding for the polypeptides disclosed, preferably such polynucleotide corresponds to SEQ ID NO 1, 3, 6, 8, 10.

A)



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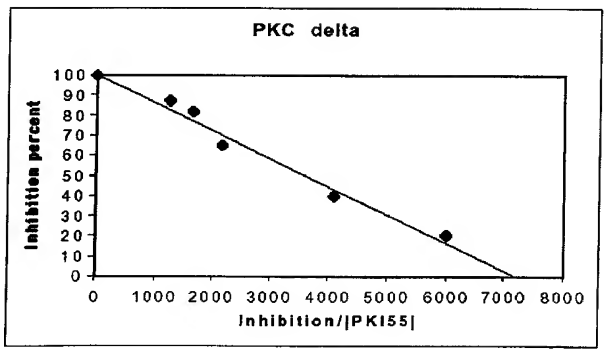

WO 03/080662 A1

PROTEIN KINASE C MODULATORS, THEIR AMINO ACID AND NUCLEOTIDE SEQUENCES AND USES THEREOF

DESCRIPTION

Field of the invention

The present invention provides novel polynucleotides and proteins encoded by such polynucleotides capable of modulating the Protein Kinase C activity for therapeutic, diagnostic and research purposes.

The present invention provides novel polynucleotides and proteins encoded by such polynucleotides produced along the PKC pathway that act as PKCmodulators.They have a low affinity and must accumulate in the cell before reaching the critical concentration for inhibiting the activated enzyme.The resulting feed-back mechanism prevents the expression of overactivated pkc that is implicated in a number of important human diseases including cancer, diabetes and disorders of the immune system.

Background

Protein Kinase C (PKC) is a key family of enzymes that has a central role in the "signal transduction" mechanism and is involved in many critical pathways in cell activation. It can be defined as a heterogeneous group of at least 11 phospholipid dependent serine/threonine kinases, characterized by similarity in their overall amino acid sequence. On the basis of overall amino acid sequence similarity, the PKC family has been divided into subfamilies or subtypes. The first subfamily which is usually referred as the classical or conventional PKCs (cPKCs) consists of PKC-α, βI, βII and γ. A second subfamily (so called novel PKCs or nPKCs) comprises PKC-δ, ε, η and θ and a third one (atypical PKCs) contains PKC-ζ and τ. PKC-λ falls within this last group as well but appears to be the mouse homolog of PKC-τ.

The alignment of the various PKC isotypes allows the identification of several highly conserved regions. PKC "isozymes" consist of a single polypeptide chain with an aminoterminal regulatory region (20-70 kDa) and a carboxy terminal catalytic region (45 kDa) (4-5), linked up by a hinge. While the catalytic regions of the PKCs show a high degree of homology, the regulatory regions are more diverse and indeed the diversity in the regulatory regions provides the basis for the

subdivision of the PKC family in the different subgrops as mentioned before. More particularly the regulatory region contains an autoinhibitory domain, which acts like a pseudosubstrate, and C1, present in all isozymes and C2 present in either conventional PKC (cPKCs) or novel PKCs (nPKCs). The catalytic region contains C3 and C4 domains that comprise the ATP, and substrate binding lobes of the kinase core and are present in all isozymes.

C1 domain binds diacylglycerol (DAG) or phorbol esters, C2 binds phosphatidylserine (PS) and, in cPKCs, Ca^{2+} ions. cPKCs (isotypes α, βI, βII, γ) are regulated by PS, Ca^{2+} and DAG or phorbol esters, as cofactors. nPKCs (isotypes δ, ε, η, θ) do require only DAG and phorbol esters. In the cPKCs calcium ions binding leads to a change in the active conformation of the enzyme, through the coordination of the C2 domain (6), so that the domain is readied for binding to lipids; according to the present invention the conformationally modified PKC is the target of PKI55. In the nPKCs, C2 domain is already structured to bind lipids and does not require Ca^{2+} (7). Atypical PKCs isotypes (ξ, σ, λ) are not activated by Ca^{2+} and DAG in vitro, but they are stimulated by PS.

Activation of PKCs follows the removal of the autoinhibitory pseudosubstrate domain from the active site, that is achieved by a conformational change induced by highly specific binding of DAG and PS to the two membrane-targeting domains C1 and C2. Under normal conditions, PKC activation is reversible: DAG is rapidly metabolized, the Ca^{2+} concentration is reduced and the PKC becomes active, only for the time that is necessary to produce an enzymatic cascade of a signal transduction pathway. This is the most widely accepted model to describe the regulation of PKC through the reversible activation of co-factors (7). Whenever activators are produced for longer periods of time and/or their degradation is reduced, as in the case of Phorbol Esters treatment or, even under physiological condition (8), PKC activation is extended. In such a situation, the simple removal of modulators may not be sufficient to prevent proliferation, growth and differentiation effects which cannot be controlled (8,9,41). It is known that protein kinases over-expression is implicated in a number of important human diseases including cancer, diabetes and disorders of the immune system.

In the early days of PKC research it was very soon evident to researchers that the

therapeutic possibilities for PKC inhibitors were endles but that the flip side of the coin was that the toxicity penalties associated with these agents might be so great as to preclude their therapeutic potential (38).

Several inhibitors have been studied that recognize as target either the regulatory domain (Phospholipids and phorbol ester binding sites) or catalitic domains (peptide and ATP binding sites). However toxicity prevented a wide use of synthetic inhibitors for therapeutic purposes.

Antisense technology and mutated inactive kinase have been used to develop specific isoenzyme inhibitors. However limitations to these methods have been encountered: the main difficulty lies in similarities of sequences in the various domains of the different isozymes so that specific inhibitors remain desiderable.

Several peptide inibitors have been derived from the pseudosubstrate sequence, given the fact that this sequence is inserted in the inactive form of the enzyme in the active site it was a natural candidate for "peptide inhibition" of the enzyme itself. These peptide inhibitors that bind to peptide substrate binding site on PKC, inactivate the enzyme. They are powerful and highly selective. However their usefulness is limited, because they cannot penetrate the cell membrane.

Chronic stimulation of PKC leads to a marked decrease in the cellular content of most PKC isotypes: this phenomenon has been termed downregulation and desentitize the cell to a subsequent PKC-mediated signal for a certain period suggesting a modulatory adaptive function.

Summary of the invention

A 168 bp amplification product was obtained in RT-PCR experiments using a degenerate oligonucleotide designed on a five AA sequence of IN, a 7kDa protein, previously characterized as PKC inhibitor. It was included in the coding ORF of the 1530 bp long I.M.A.G.E. clone ID 38900 (accession numbers R51337 and R51448) that produces a translation product of 6.5 KDa.

The translation of the ORF conceptual reading frame allowed the preparation of the synthetic protein PKI55 that was found to inhibit and degrade both untreated nPKC d isozymes and activated cPKC isozymes. PKI55 gene is localized in chromosome 2q35. The Repeat Maskers output showed a 533 bp-long LTR32/ERVL segment that included the PKI55 coding sequence and a complete

regulatory region. The coding sequence and the structure of PKI55 were detected in a cDNA brain of Macaca Fascicularis (diverged from human lineages about 25 Myr ago), Three other human genes with over 55% identities with PKI55 were identified in three different loci (i.e. chromosomes 10, 15 and 20.) Synthesis of PKI55 was stimulated by PKC activation. A feedback loop of inhibition is established. When the PKCs are over-activated, PKI55 induces degradation of the enzyme and prevents the isozyme over-expression implicated in a number of important diseases including cancer, diabetes and disorders of the immune system. The presence of the PKI55 sequence in Macaca Fascicularis as well as in human chromosomes 10, 15 and 20 indicates a selective advantage for the PKI55 sequence and the adaptive value of the feedback mechanism(42).

The main object of the present invention is related to a new family of Protein Kinase C modulator, polypeptidic in nature, characterized by their ability to bind to Protein Kinase C. Such modulators bind to the alpha, beta I, beta II, gamma, delta, epsilon, lambda, theta, mu, eta and zeta PKC isoforms. The polypeptides according to the invention bind Protein Kinase C when this is in an activated form. According to preferred embodiments such polypeptides correspond to SEQ ID NO 2, 4, 5, 7, 9, 11, 12 and to polypeptides showing a homology to the sequence set forth in SEQ ID NO: 2 comprised in the range from at least 40% to 99% to the sequence set forth in SEQ ID NO: 2. Also the invention refers to functional subsequence of these polypeptides, chosen among peptides listed in the following "PKC isolation and Assay" and to peptides carrying aminoacid substitution, deletion or insertion with respect to the same.

According to a further embodiment the invention relates to chimaeric proteins carrying heterologous aminoacid sequences at the N- or C-terminal of SEQs 2, 4, 5, 7, 9, 11, 12 or to their functional subdomains.

According to a further embodiment the invention comprises antibodies or antibody-fragment thereof that specifically binds to the polypeptides of SEQ ID NO 2, 4, 5, 7, 9, 11, 12 .

The invention also provides for the nucleotidic sequence encoding for the polypeptides disclosed, preferably such polynucleotide corresponds to SEQ ID NO 1, 3, 6, 8, 10. Also provided are homologous polynucleotidic sequences showing a

degree of homology of at least 50%, or showing hybridization to SEQ ID NO 1, 3, 6, 8, 10 and RNA, complementary sequences, genomic and cDNA sequences encoding the polypeptides set forth in SEQ ID NO 2, 4, 5, 7, 9, 11, 12 and to their fragments.

Polypeptides and polynucleotides according to the invention are used in the pharmaceutical field both for therapeutic as well as for diagnostic purposes. According to a preferred embodiment, they are comprised in pharmaceutical composition for the therapy, prevention and diagnosis of diseases characterized by PKC disregulation.

The compositions of the present invention include: isolated proteins comprising the indicated polypeptides, isolated polynucleotides encoding such polypeptides, including recombinant DNA molecules, cloned genes or degenerate variants thereof, especially naturally occurring variants such as allelic variants, and antibodies that specifically recognize one or more epitopes present on such polypeptides.

The compositions of the present invention additionally include vectors, including expression vectors, containing the polynucleotides of the invention; cells genetically engineered to contain such polynucleotides and cells genetically engineered to express such polynucleotides.

The compositions of the present invention may further comprise an acceptable carrier, such as pharmaceutically acceptable carrier.

The invention also relates to methods for producing a polypeptide comprising growing a culture of the cells of the invention in a suitable culture medium, and purifying the protein from the culture. Preferred embodiments include those in which the protein produced by such process is a mature form of the protein.

According to a further embodiment the invention also provides methods for the identification of compounds that modulate the expression of the polynucleotides and/or polypeptides of the invention or that are able to inhibit the binding between PKC and the peptides of the invention.

Polynucleotides according to the invention have numerous applications in a variety of techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use as oligomers for PCR, use for

chromosome and gene mapping, use in the recombinant production of protein, and use in generation of anti-sense DNA or RNA, their chemical analogs and the like. For example, when the expression of an mRNA is largely restricted to a particular cell or tissue type, polynucleotides of the invention can be used as hybridization probes to detect the presence of the particular cell or tissue mRNA in a sample using, e.g., in situ hybridization.

In other exemplary embodiments, the polynucleotides are used in diagnostics as expressed sequence tags for identifying expressed genes or, as well known in the art and exemplified by (39), as expressed sequence tags for physical mapping of the human genome.

The polypeptides according to the invention can be used in a variety of conventional procedures and methods that are currently applied to other proteins. For example, a polypeptide of the invention can be used to generate an antibody that specifically binds the polypeptide.

According to another example the polypeptides according to the invention can be used for drug design in a method comprising the following steps: To obtain highly purified complex between the PKC and PKI55 (or any other relevant member of the family) in order to produce high quality crystals /co-crystals in case of the x-rays crystallography approach is selected or to proceed to the study of the complex structure with the NMR approach; to use the information on the structure of the complex between the two proteins to obtain a fine definition of the regions of the to molecules involved in the complex formation; to use the map of these binding sites of the two molecules to model (or select in a library) synthetic molecules mimicking the binding and the action of the PKI55 (or any other member of the family); to evaluate the action of the selected lead/s in vitro PKC assay and in relevant in vivo model to evaluate the in vivo action; to proceed to an evaluation of the SAR (structure activity relationships) to optimize the identified lead/s; to proceed to optimized lead development process.

Methods are also provided for preventing, treating or ameliorating a medical condition, which comprises administering to a mammalian subject a therapeutically effective amount of a composition comprising a protein of the present invention and a pharmaceutically acceptable carrier. Liposomes are also enclosed.

In particular, the polypeptides and polynucleotides of the invention can be utilized for the tratment of any disease in the patogenesis or clinical manifestation of which the disregulation of PKC is involved. The polypeptides and polynucleotides of the invention may, therefore, be utilized, for example, as part of methods for treatment in disease areas including but not limited to: Oncology, Neurology, Inflammation, Allergy and Autoimmune Disorders.

Certainly the ability to modulate the activity of PKC can be very important in all those diseases in which the PKC is too active or activated for a too long time period.

The methods of the present invention further relate to methods for detecting the presence of the polynucleotides or polypeptides of the invention in a sample. Such methods can, for example, be utilized as part of prognostic and diagnostic evaluation of disorders as recited above and for the identification of subjects exhibiting a predisposition to such conditions.

The complex structure of PKI55 is certainly prone to mutations that may affect the expression of the protein (regulatory mutations) as well as structural mutations that may affect the ability of the mutated protein to bind to activated PKC and perform its modulatory function.

The inventors have obtained data from patients cells showing that in cells from patients with rinithis the protein is totally undetectable suggesting that a mutation to promoter or other regulatory regions may interfere with the expresssion of the modulatory protein potentially exposing the cell to the consequences of a long period of PKC activation.

The knowledge of these mutations as well known in the art may be useful to diagnosis of diseases or of risk of contracting the disease when the individual bearing that mutation is exposed to the causing environmental factor (40)

Furthermore, the invention provides methods for evaluating the efficacy of drugs, and monitoring the progress of patients, involved in clinical trials for the treatment of disorders as recited above. Accordingly the polypeptides of the invention can be used for diagnostic uses in a method comprising the following steps: to compare the KI55 (or any other member of the family relevant) sequence in healthy subjects and in-patients affected by disease/s possibly related to a PKI55 (or analogous)

deficit or impaired function; to identify mutations (or deletions or any other rearrangement) causing either an abnormal protein structure or a disregulation of the protein itself expression; to prove the clinical relevance of the identified mutation/s (or deletions or any other rearrangement) with one or more clinical case-control or family studies demonstrating a statistically significance difference between carriers of the mutation/s and controls in term of increased risk for the relevant disease; to select the most practical available technology for a diagnostic kit to be prepared.

The invention also provides methods for the identification of compounds that modulate the expression. Such methods can be utilized, for example, for the identification of compounds that can ameliorate symptoms of disorders as recited above. Such methods can include, but are not limited to, assays for identifying compounds and other substances that interact with (e.g. bind to) the polypeptides of the invention. Compounds mimicking the biological activityof PKI55 or compounds that can bind to the activated PKC producing at molecular level effects similar to those resulting from the binding of PKI55 and causing an increased level of proteolysis of the enzyme. These compounds may be of therapeutic interest in those conditions in which an abnormal or too long activation of PKC is crucial in the pathogenesis process . . .

The methods of the invention also include methods for the treatment of disorders as recited above which may involve the administration of such compounds to individuals exhibiting symptoms or tendencies related to disorders as recited above. In addition, the invention encompasses methods for treating diseases or disorders as recited above by administering compounds and other substances that modulate the overall activity of the target gene products. Compounds and other substances can effect such modulation either on the level of target gene expression or target protein activity.

Further objects of the invention will become evident from the following detailed description of the invention.

DETAILED DESCRIPTION

DEFINITIONS

The term "nucleotide sequence" refers to a heteropolymer of nucleotides or the

sequence of these nucleotides. The terms "nucleic acid" and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of nucleotides. Generally, nucleic acid segments provided by this invention may be assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, or from individual nucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon, or a eukaryotic gene.

The terms "oligonucleotide fragment" or a "polynucleotide fragment", "portion," or "segment" is a stretch of polypeptide nucleotide residues which is long enough to use in polymerase chain reaction (PCR) or various hybridization procedures to identify or amplify identical or related parts of mRNA or DNA molecules.

"Oligonucleotides" or "nucleic acid probes" are prepared based on the polynucleotide sequences provided in the present invention. Oligonucleotides comprise portions of such a polynucleotide sequence having at least about 15 nucleotides and usually at least about 20 nucleotides. Nucleic acid probes comprise portions of such a polynucleotide sequence having fewer nucleotides than about 6 kb, usually less than about 1 kb. After appropriate testing to eliminate false positives, these probes may, for example, be used to determine whether specific mRNA molecules are present in a cell or tissue or to isolate similar nucleic acid sequences from chromosomal DNA as described by Walsh et al. (Walsh, .p .S. et al., 1992, PCR Methods Appll:241-250).

The term "probes" includes naturally occurring or recombinant or chemically synthesized single- or double-stranded nucleic acids. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes of the present invention, their preparation and/or labeling are elaborated in Sambrook, J. et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY; or Ausubel, F .M. et al., 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York NY, both of which are incorporated herein by reference in their entirety.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Stringent conditions can include highly stringent conditions

(i.e., hybridization to filter-bound DNA under in 0.5 M NaHPO4, 7% sodium dodecyl sulfate (SDS), 1 mM EDT A at 65° C, and washing in 0.1xSSC/0.1% SDS at 68° C), and moderately stringent conditions (i.e., washing in 0.2xSSC/0.1% SDS at 42° C).

In instances wherein hybridization of deoxyoligonucleotides is concerned, additional exemplary stringent hybridization conditions include washing in 6xSSC/O.O5% sodium pyrophosphate at 37°C (for l4-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos).

As used herein, the terms"polypeptide", "protein"and"peptide"are used almost interchangeably to denote two or more amino acids covalently linked by an amide bond or equivalent with different length. The polypeptides of the invention are of unlimited length and may comprise L- and D- isomers and combinations thereof. Such polypeptides can include modifications typically associated with post-translational processing of proteins, for example, cyclization (e. g., disulfide bond), phosphorylation, glycosylation, carboxylation, ubiquitination, or lipidation. Polypeptides of the invention further can include compounds having amino acid structural and functional analogues, for example, peptidomimetics having synthetic or non-natural amino acids or amino acid analogues, so long as the mimetic has one or more functions or activities of Protein Kinase C modulators as set forth herein. Non-natural and non-amide chemical bonds, and other coupling means can also be included, for example, glutaraldehyde, N-hydoxysuccinimide esters, bifunctional maleimides, or N, N'-dicyclohexylcarbodiimide (DCC). Non-amide bonds can include, for example, ketomethylene aminomethylene, olefin, ether, thioether and the like (see, e. g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide and Backbone Modifications,"Marcel Decker, NY).

In yet another embodiment, the invention provides functional subsequences of PKI55 and its homologous or paralogous or orthologous sequence, including their peptidic fragments. Functional subsequences include portions of PKI55 and homologous polypeptides having 40% or more, 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, and 95% or more identity to the sequences set forth in SEQ ID NO: 2, 4, 5, 7, 9, 11, as well as functional subsequences of

SEQ ID NO: 2, 4, 5, 7, 9, 11.

As used herein, the term "subsequence" means a sequence region or portion of PKI55 polypeptide or polynucleotide (e.g., SEQ ID NO: 2 or SEQ ID NO: 1). "Functional subsequence" means a subsequence that has one or more functions or activities of exemplary PKI55 polypeptide, as described herein. For example, polypeptide binding to PKC, can modulate activity of JNK, AP-1 or NF-KB, or production of IL-2.

As used herein, the term "isolated", when used as a modifier of invention polypeptides, antibodies, polynucleotides and cells described herein, means that the compositions are made by the hand of man and are separated from their naturally occurring in vivo environment. Generally, the compositions so separated are substantially free of one or more other proteins, polynucleotides, lipids, carbohydrates, or other materials with which they may normally associate with in nature. An"isolated"polypeptide, antibody or polynucleotide can also be "substantially pure"when free of most or all of the materials with which they may normally associate with in nature. Thus, an isolated compound that also is substantially pure does not include polypeptides or polynucleotides present among millions of other sequences, such as nucleic acids in a genomic or cDNA library, for example. Typically, the purity can be at least about 60% or more by mass. The purity can also be about 70% or 80% or more, and can be greater, for example, 90% or more. Purity can be determined by any appropriate method, including, for example, UV spectroscopy, chromatography (e. g., HPLC, gas phase), gel electrophoresis and sequence analysis (nucleic acid and peptide).

The term "recombinant", when used herein to refer to a polypeptide or protein, means that a polypeptide or protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, e.g., E.Coli will be free of glycosylation modifications; polypeptides or proteins expressed in yeast will have a glycosylation pattern in general different from those

expressed in mammalian cells:

The term "recombinant expression vehicle or vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. An expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

The term "recombinant expression system" means host cells, which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit extrachromosomally. Recombinant expression systems as defined herein will express heterologous polypeptides or proteins upon induction of the regulatory elements linked to the DNA segment or synthetic gene to be expressed. This term also means host cells, which have stably integrated a recombinant genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers. Recombinant expression systems as defined herein will express polypeptides or proteins endogenous to the cell upon induction of the regulatory elements linked to the endogenous DNA segment or gene to be expressed. The cells can be prokaryotic or eukaryotic.

The term "open reading frame", ORF, means a series of nucleotide triplets coding for amino acids without any termination codons and is a sequence translatable into protein.

The term "expression modulating fragment," EMF, means a series of nucleotides which modulates the expression of an operably linked ORF or another EMF.

As used herein, a sequence is said to "modulate the expression of an operably linked sequence" when the expression of the sequence is altered by the presence

of the EMF. EMFs include, but are not limited to, promoters, and promoter modulating sequences (inducible elements). One class of EMFs are fragments which induce the expression or an operably linked ORF in response to a specific regulatory factor or physiological event.

As used herein, an "uptake modulating fragment," UMF, means a series of nucleotides which mediate the uptake of a linked DNA fragment into a cell. UMFs can be readily identified using known UMFs as a target sequence or target motif with the computer-based systems described below.

The presence and activity of a UMF can be confirmed by attaching the suspected UMF to a marker sequence. The resulting nucleic acid molecule is then incubated with an appropriate host under appropriate conditions and the uptake of the marker sequence is determined. As described above, a UMF will increase the frequency of uptake of a linked marker sequence.

The term "active" refers to those forms of the polypeptide which retain the biologic and/or immunologic activities of any naturally occurring polypeptide.

The term "naturally occurring polypeptide" refers to polypeptides produced by cells that have not been genetically engineered and specifically contemplates various polypeptides arising from post-translational modifications of the polypeptide including, but not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

The term "derivative" refers to polypeptides chemically modified by such techniques as ubiquitination, labeling (e.g., with radionuclides or various enzymes), pegylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins.

The term "recombinant variant" refers to any polypeptide differing from naturally occurring polypeptides by amino acid insertions, deletions, and substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as cellular trafficking, may be found by comparing the sequence of the particular polypeptide with that of homologous peptides and minimizing the number of amino acid sequence changes made in regions of high homology.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

Alternatively, where alteration of function is desired, insertions, deletions or non-conservative alterations can be engineered to produce altered polypeptides. Such alterations can, for example, alter one or more of the biological functions or biochemical characteristics of the polypeptides of the invention. For example, such alterations may change polypeptide characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate. Further, such alterations can be selected so as to generate polypeptides that are better suited for expression, scale up and the like in the host cells chosen for expression. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges.

As used herein, "substantially equivalent" can refer both to nucleotide and amino acid sequences, for example a mutant sequence, that varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences.

The term "identity" is referred to the same nucleotide or amino acid unit in the whole sequence length considered.

Typically, such a substantially equivalent sequence varies from one of those listed herein by no more than about 30%, i.e. the number of individual residue substitutions, additions, and/or deletions in a substantially equivalent sequence, as compared to the corresponding reference sequence, divided by the total number of residues in the substantially equivalent sequence is about 0.2 or less). Such a sequence is said to have 70% sequence identity to the listed sequence. In one embodiment, a substantially equivalent, mutant, sequence of the invention varies from a listed sequence by no more than 10% (90% sequence identity); in a variation of this embodiment, by no more than 5% (95% sequence identity); and in a further variation of this embodiment, by no more than 2% (98% sequence identity). Substantially equivalent, mutant, amino acid sequences according to the invention generally have at least 95% sequence identity with a listed amino acid sequence, whereas substantially equivalent nucleotide sequence of the invention can have lower percent sequence identities, taking into account, for example, the redundancy or degeneracy of the genetic code. For the purposes of the present invention, sequences having substantially equivalent biological activity and substantially equivalent expression characteristics are considered substantially equivalent.

Nucleic acid sequences encoding such substantially equivalent sequences, sequences of the recited percent identities can routinely be isolated and identified via standard hybridization procedures well known to those of skill in the art.

Where desired, an expression vector may be designed to contain a "signal or leader sequence" which will direct the polypeptide through the membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

A polypeptide “fragment”, "portion" or “segment” is a stretch of amino acid residues of at least about 4 amino acids, preferably 5, more preferably 6, and typically at least about 9 to 13 amino acids, and, in various embodiments, at least about 17 or more amino acids. When functional, any polypeptide must have sufficient length to display a biologic and/or immunologic activity.

Alternatively, recombinant variants encoding these same or similar polypeptides

may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes, which produce various restriction sites, may be le introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations in the polynucleotide sequence may be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide, to change characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate.

The term "activated" cells as used in this application are those which are engaged in extracellular or intracellular membrane trafficking, including the export of neurosecretory or enzymatic molecules as part of a normal or disease process.

The term "purified" as used herein denotes that the indicated nucleic acid or polypeptide is present in the substantial absence of other biological macromolecules, e.g., polynucleotides, proteins, and the like. In one embodiment, the polynucleotide or polypeptide is purified such that it constitutes at least 95% by weight, more preferably at least 99.8% by weight, of the indicated biological macromolecules present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000 dalton, can be present).

The term "isolated" as used herein refers to a nucleic acid or polypeptide separated from at least one other component (e.g., nucleic acid or polypeptide) present with the nucleic acid or polypeptide in its natural source. In one embodiment, the nucleic acid or polypeptide is found in the presence of (if anything) only a solvent, buffer, ion, or other components normally present in a solution of the same. The terms "isolated" and "purified" do not encompass nucleic acids or polypeptides present in their natural source.

The term "infection" refers to the introduction of nucleic acids into a suitable host cell by use of a virus or viral vector.

The term "transformation" means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

The term "transfection" refers to the taking up of an expression vector by a suitable host cell, whether or not any coding sequences are in fact expressed.

The term "intermediate fragment" means a nucleic acid between 5 and 1000 bases in length, and preferably between 10 and 40 bp in length.

The term "secreted" protein includes a protein that is transported across or through a membrane, including transport as a result of signal sequences in its amino acid sequence when it is expressed in a suitable host cell. "Secreted" proteins also includes without limitation proteins secreted wholly (e.g., soluble proteins) or partially (e.g., receptors) from the cell in which they are expressed. "Secreted" proteins also include without limitation proteins, which are transported across the membrane of the endoplasmic reticulum. "Secreted" proteins are also intended to include proteins containing non-typical signal sequences (e.g. Interleukin-I Beta, see Krasney, p .A. and Young, p .R. (1992) C.ytokine 4(2): 134 -143) and factors released from damaged cells (e.g. Interleukin-I Receptor Antagonist, see Arend, W.P. et. al. (1998). Immunol. 16:27-55).

The term "ortholog genes" includes DNA sequences that are derived from the same gene through speciation i.e. two orthologs are two analogous or similar genes in two different species

The term "paralogous genes" are genes that are derived from a gene duplication i.e. two or more genes very similar in the same species.

Each of the above terms is meant to encompass all that is described for each, unless the context dictates otherwise.

Abbreviations: used in the present description CEPH, Centre d'Etude du Polymorphisme Humain; YAC, Yeast Artificial Chromosome; I.M.A.G.E., Integrated Molecular Analysis of Genomes and their Expression; dbEST, database Expressed Sequence Tag.

PROTEINS

According to a first embodiment the invention provides a family of polypeptidic Protein Kinase C modulators, called PKI55 (Protein Kinase Inhibitor 55) or EK-MP (Endogenous Kinases-Modulators Proteins).

The class of PKI55 modulator works differently from all known PKC inhibitors, as they bind to PKC, modulating its activity. The modulators according to the present invention are sensitive to the conformation of their target, as they bind only to activated PKC. Thus, only PKC enzyme activated molecules are recognized by the

modulators of the present invention and the remaining ones do not represent targets.

According to a preferred embodiment of the invention such modulators bind preferably to PKC isoforms such as: alpha, beta I, beta II, gamma, delta, epsilon, lambda, theta, mu, eta and zeta, more preferably to PKC isoforms alpha and delta.

The new modulators identified in the present invention binds to PKC only after the kinase has been activated with a conformational change by one of the known activators such as: Ca^{++}, diacilglicerolo (DAG), phorbol esters, phosphatidil serine (PS) or other specific or aspecific activators.

This binding, which has an affinity constant in the micromolar range, preferably comprised between 5 μM and 50 μM, is extremely useful to attain a fine downregulation only of activated PKC. The relatively low affinity of binding is in agreement with the molecular structure of PKI55 obtained by NMR studies (Fig. 4). It allows accumulation of PKI55 to a critical concentration and the activated enzyme is inhibited and degraded. Due to the PKC dependence of the PKI55 synthesis de novo the low affinity is the essential step in the feedback mechanism that does not affect the catalytic activity of PKC indispensable for normal life of the individual but prevents the toxic effects of PKC overexpression (43).

Chronic activation of PKC leads to downregulation by reduction in the cellular content of most PKC isozymes, followed by desensitization, that is the inability of the cell to respond to subsequent PKC stimulation. Desensitization is known to occur after prolonged PKC activation.

The Authors have shown that the observed downregulation is the effect of the action of proteases, preferably calpain. The conformational change associated with PKC activation allows its binding to the modulators of the present invention and this binding enhances the PKC susceptibility to proteolysis in the hinge region, which is effected by proteases and which leads to the formation of two fragments: one, which is 50 kDa in size, which is endowed with a constitutive catalytic activity (PKM); the other, which measures 30 kDa, binds the phorbol esters. Both fragments are subsequently completely degraded.

The demonstration that downregulation reflects an increase in the rate of degradation, more than in the rate of PKC synthesis, indicates a "non-signaling"

role just like that of down regulation associated with desensitization (10).

The inventors have described a feedback mechanism for downregulation of the activity of PKC based on the protein PKI55 that binds to PKC with a suicide inhibitory mechanism. A dis-regulation or a defect in the discovered mechanism is very important in many diseases in which a defect in signal transduction mechanism may be crucial: these include, non-exhaustively: autoimmune and allergic diseases, oncologic and neurologic diseases.

The importance of this PKC regulation mechanism is confirmed by the fact that it is based on the preservation of the gene structure since 25Myr after the divergence of Human and Old World Monkeys Lineage.

The PKI55 class of inhibitors associates with its target in an irreversible manner. This behaviour is similar to that of a suicidal inhibitor, which is required when a harmful "substance" has to be eliminated.

The increased susceptibility to proteolitic digestion of PKC, as the result of the PKI55 modulator binding, is a mechanism common the one used by the cell in biological processes such as the removal of harmful elements, such as oncogene-like elements: since PKI55 is a proteolysis- resistant molecule, this allows for its re-utilization and an almost catalytic function in this regard.

However, without being linked to a specific mechanism of action the invention provides for polypeptide modulators which upon binding to PKC, determine a slow reduction/increase in the known signal transduction pathways of PKC.

Both because of the presence of many isoforms and because of its molecular characteristics, PKC is involved in a great number of essential cell functions, which need not be regulated at the same time and at the same rate. The availability of a "slow" modulator, therefore, becomes an advantage. PKI55, with its affinity for PKC in the micromolar range, probably fulfills such a function, succeeding in turning off the different PKC activities in a gradual manner, enabling the cell to carry on some functions but not others.

Of note, a peculiarity of the modulators of the present invention is the graduality of the effects evident in relation to the PKC activation time length.

PKC, activated by cofactors, is effective for a short time and rapidly returns to an inactive state following the decay of the effectors; in this situation the PKC-PKI55

complex is already visible, but the physiological function of PKC is not affected. Aliquots of PKC isozymes in the complex are however prevented from being active and are downregulated. The control of potential isozymes over activity may be considered the crucial selective advantage of PKI55.

When PKC activation is prolonged, de novo synthesis of PKI55 also increases up to a critical concentration, sufficient to downregulate all activated isozymes. After 72 hrs, in PMA activated PBMC cultures a significant increase of PKM,is detectable.

Our results show that all PKC isozymes, preferably alpha and delta isoforms, activated and therefore conformationally modified, are recognized by PKI55 that, at the proper critical cellular concentration, determines PKC desensitization.

PKI55 appears to be biologically significant as potential modulator of cellular PKC Ensuring an optimal running level of the enzyme may represent the selective advantage of the evolutionarily conserved KI55 gene.

According to a preferred embodiment represented by an in vitro assay illustrated in ab. 2 we show that the removal of cofactors such as Calcium, Diacylglycerol, phosphatidilserine and ATP reduces the V_{max} observed, but leaves unchanged the percentage of PKC inhibition, supporting the idea that a specific conformation is the target recognized by PKI55. Removal of Ca^{2+} from the mixture prevents the cPKCα conformational change and the isozyme becomes insensitive to PKI55, while it leaves practically unchanged the nPKCδ isozyme sensitivity to PKI55.

PKI55 interacts with conformationally adapted PKC molecule, forming a 1:1 irreversible complex that can be directly demonstrated according to methods known in the art, i.e. by coprecipitation. The binding is irreversible, also if Ca^{2+} ions are removed or PKC inhibitor is added. Furthermore, the PKC in the complex becomes more sensitive to inactivation and degradation into PKM by protease, preferably calpain.

Acccording to this embodiment, the modulators of the present invention contributes to degradation of activated PKCs, ending in PKC downregulation.

Thus, PKI55 acts as a mechanism-based modulator, which is relatively inactive until the enzyme attains an active conformation; it is not modified by the enzyme but, rather, combines irreversibly with it and allows its degradation by proteolysis.

Cell stimulation induces synthesis of the PKI55 (6.5 kDa) that is suppressed by cycloexamide, indicating that these inhibitors are synthetized de novo.

Cell treatment with the specific PKC inhibitor, such as H7 ([1-(5-isoquinoline sulfonyl)-2-methylpiperazine2HCl] Quick J. et al 1992,B.B.R.C 187,657), suppresses the synthesis de novo of PKI55. This finding suggests that de novo synthesis of PKI55 occurs along the PKC pathway. PKI55 in turn forms also in vivo, an irreversible complex with conformationally modified PKC, where the enzyme is inhibited and degraded. These results show that PKC modulation is regulated at least by a particular back-inhibition loop, in activated PKC, which stimulates PKI55 synthesis and that the protein, in turn, inhibits PKC through the formation of an irreversible complex.

According to a particularly preferred embodiment, the modulator according to the present invention has the aminoacid sequence corresponding to SEQ ID NO 2 and is characterised by a molecular weight of about 6.5kd as determined by SDS-PAGE. However, the invention also encompasses allelic variants of the disclosed polypeptides or proteins; that is, naturally-occurring alternative forms of the isolated polypeptides which are identical, homologous or related to that encoded by the polynucleotide disclosed in the present application.

Species or tissue specific analogues or PKC subunits specific inhibitors whose encoding sequences may be isolated on the basis of the homology to PKI55 (i.e. by direct sequence comparison or by hybridization or PCR techniques or by database screening), are also encompassed by the present invention.

For example according to this embodiment, the invention provides for the aminoacid and nucleotidic sequences or a class of polypeptidic PKC modulator all sharing the same mechanism of PKC regulation and an aminoacid homology, preferably identity, to SEQ ID NO: 2, 4, 5, 7, 9, 11, 12, 13 enclosed in the sequence listing of at least about 55%, more typically at least about 70%, or even more preferably 80%, 90%, 95% or more preferably 98%, comprising the intermediate values.

Moreover the invention provides for the aminoacid sequence of orthologue sequence such as the one identified in M. fascicularia corresponding to the protein encoded by SEQ ID NO 10 and for the aminoacid sequence of three human

paralogue sequences identified on chromosomes 10, 15, 20 and corresponding to the sequences encoded by SEQ ID NO 3, 6, 8 and to their conservative mutant, allelic variant, or aminoacidic sequences sharing a degree of homology, preferably identity, of at least about 55%, more typically at least about 70%, or even more preferably 80% and 90%, 95%, 98%, comprising the intermediate values. Notably the PKC-modulators of he present invention are endowed with the ability to crossing the cell membrane and modulating PKC-activity in PMBC lymphocytes, when added to the culture medium in concentration of at least 1 μM.

According to a further aspect the present invention encompasses fragments of the peptidic modulator disclosed, preferably maintaining the same biological activity and specificity of binding of the entire molecule and called therefore functional fragments.

Such fragments are identified starting from the entire sequence SEQ ID NO 2 disclosed in the present invention, and homologous sequences thereof according to methods known in the art. Methods comprising i.e. the proteolytic cleavage of the molecule, the isolation of the fragments followed by assaying their biological activity in a direct ligand binding assay in the presence of PKC, or in competition assays in the presence of PKC and of the entire modulator molecule.

Also the identification of the active peptides in the complete sequence is obtained by chemical synthesis of overlapping peptides comprising at least 4 aminoacids more preferably a number of aminoacids comprised from 6 to 20 and spanning the entire PKI55 molecule. Chemical synthesis is performed by known methods: once the peptides are obtained they are used in the assays described before to verify their ability to bind PKC.

In a preferred embodiment the peptides according to the invention comprise the N-terminal region of PKI55 inhibitors and span at least residue 1-30 of SEQ ID NO 2, more preferably res. 1-26 (SEQ ID NO 12), even more preferably residue 1-16 (SEQ ID NO 13). According to this embodiment peptides are preferably chosen among:

1-4: MLYK

5-10: LHDVCR

1-10: MLYKLHDVCR

1-16: MLYKLHDVCRQLWFSC

1-26: MLYKLHDVCRQLWFSCPACHHRAMRI

The invention also provides for mutants of such fragments preferably maintaining the same biological activity and binding affinity of the corresponding non mutated peptide.

In addition, and according to this embodiment, the invention comprises the N-terminal region of any ortholog and paralog identified by the present invention, which is different in the amminoacidic sequence, and which comprises at least 4 aminoacids, more preferably a number of aminoacids comprised from 6 to 20, derived from the N-terminal region of sequences ID NO 2, 4, 5, 7, 9, 11.

The most preferred peptide is peptide 1-16 (SEQ ID NO 13).

The peptides disclosed in the present invention may be produced by chemical synthesis or by recombinant DNA methods. They are rendered more stable by modifications obtained according to methods known in the art: i.e. they may be amidated at the C-terminus to avoid carboxypeptidase degradation, or they may be dimetilated at the N-terminus to preserve this end. Also comprised in the scope of the present invention are peptides with the aminoacid sequence corresponding to sequences 2, 4, 5, 7, 9, 11, 12, 13 or their fragments thereof, comprising one or more aminoacids in D-configuration with the aim of obtaining protease-stable molecules.

Fragments of the proteins disclosed, may be in linear form or they may be cyclized using known methods, for example, as described in H. U. Saragovi, et al., Biotechnology 10,773-778 (1992) and in R. S. McDowell, et al., J. Amer. Chem. Soc. 114, 9245-9253 (1992), both of which are incorporated herein by reference.

The polypeptides disclosed in the present invention and their fragments thereof, may be also fused to carrier molecules such as immunoglobulins for many purposes, including for increasing the valency of protein binding sites by recombinant of chemical methods known in the art. For example, fragments of the protein may be fused through "linker" sequences to the Fc portion of an immunoglobulin. For a bivalent form of the protein, such a fusion could be to the Fc portion of an IgG molecule. Other immunoglobulin isotypes may also be used to generate such fusions. For example, a protein-IgM fusion would generate a

decavalent form of the protein of the invention.

The present invention also provides for secreted form of the modulator which are obtained by linking the full-length form of the protein or their fragments to a signal sequence, which directs the protein outside the cell.

According to a different embodiment the invention provides for the membrane-bound forms of the polypeptide of the present invention, which comprises any modification of the aminoacidic protein, such as farnesylation, myristilation or phosphatydil-inositol, that confers modified properties of hydrophilicity, hydrophobicity or compartimentalization to the natural protein.

According to a further embodiment the invention encompasses the nucleotide sequences encoding for protein PKI55 for its paralogs and orthologs and homologus with a homology, preferably identity, of at least 55% as previously defined; allelic variant or conservative mutation, in synthesis for all the polypeptides displaying in different tissues or on different PKC isoforms or in different animal species, the same binding activity to activated PKC and the same mechanism of PKC inactivation.

Therefore according to this particular embodiment, the invention provides for a preferred nucleotide sequence encoding for human PKI55 and corresponding to SEQ ID NO 1.

However the invention encompasses all the nucleotide sequences comprising the nucleotides encoding for polypeptides corresponding to SEQ ID NO 2, 4, 5, 7, 9, 11, 12, 13 and their fragments.

In addition, the invention comprises DNA sequences having at least 55% degree homology, preferably 70%, more preferably 80%, 90%, more preferably 95%, 98% (comprising intermediate values) to SEQ ID NO 1, 3, 6, 8, 10 or sequences which hybridises in stringent conditions to the nucleotide sequences disclosed in the present invention.

This embodiment further comprises polypeptides showing only partial similarity to SEQ ID NO 2, 4, 5, 7, 9, 11, 12, 13 for example polypeptides showing a high degree of similarity in the first 20-30 aminoacid N-terminal sequences, and to the nucleotides sequences encoding such polypeptides.

The present invention in a further embodiment, also provides the sequence of the

genes from which the corresponding cDNA sequences are derived.

A preferred embodiment of the human gene encoding PKI55 modulator is represented by SEQ ID NO 1, isolated from the chromosomal region 2q35. The invention therefore provides for the coding sequence and for the regulatory regions, extending both at the 5' and comprising the promoter region as well as at the 3' end of the coding sequence, and comprising the 3' untranslated region.

The genomic organization of PKI55 is shown in Figure 3. The PKI55 gene is an intronless functional gene. Two API consensus sequences have been identified at the 5' end of the gene.

According to a further embodiment the polynucleotides of the invention comprise the regulatory regions of such modulators, namely the promoter region and the 3' untranslated region, responsible for the complex regulation involved in their de novo synthesis.

Regulatory regions for SEQ ID NO 1, are shown in Fig. 3

One of the two AP1 sequences in the promoter region may function to rescue the enhancing effect on transcription in case of inactivation or deletion of the other. Alternatively the two API consensus sequences may act synergically to improve their effect on transcription of the KI55 gene.

Indeed, one of the key properties of the enhancers is to function in either orientation and in a position independent manner, since they can be located upstream, within or downstream of the transcribed region and preferably act on the nearest promoter (31-33).

The AP1 DNA binding sites are relevant to PKI55 regulation. It is known that PKC activation leads to a phosphorylation of several substrates and results in assembling of the Fos/Jun heterodimer, which binds to AP1 elements on genes that, carry the consensus sequences proximal to the promoter; DNA binding of a functional AP1 accelerates the gene transcription. (34). AP1 consensus sequence, in the regulatory region of KI55, is consistent with the observed PKC dependence of the de novo synthesis of the protein.

Experiments of DNAse I protection, electrophoretic mobility shift and methylation interference indicate that AP1 may be critical for the expression of KI55.

In synthesis this embodiment of the invention encompasses all the sequences

which may be isolated or derived by similarity, homology studies performed by software analysis or by hybridization, or by other methods known in the art, from or using the nucleotide or aminoacid sequence information disclosed herein as SEQ ID NO 1-12 , according to methods known in the art such as the BLAST software. Said polynucleotides are of particular interest for the production of recombinant polypeptides. The derived polypeptides are in an essentially purified form which is therefore comprised in the present invention.

As a matter of fact the sequence information disclosed herein allow to confer a putative biologic function to all the sequences showing degree of homology as previously defined to the sequences provided, according to methods known in the art: such methods include the preparation of probes or primers from the disclosed sequence information for identification and/or amplification of genes in appropriate genomic libraries or other sources of genomic or cDNA materials, etc.

By providing the aminoacid and nucleotide sequences of a new class of PKC modulators, and given the crucial role of these molecules in the cell, the present invention provides also methods to identify mutations or single nucleotide polymorphisms in the coding or regulatory region of these inhibitors which can cause disease or pathologic situation. These mutations have relevance both for diagnostic as well as for therapeutic purposes.

Macaca Fascicularis brain cDNA, which represents a particular embodiment of the present invention, as the nucleotide sequence encoding for the polypeptide corresponding to SEQ ID NO 11 in the enclosed sequence listing, corresponds to a sequence identified in GenBank with accession number AB 050412.

M. fascicularia cDNA shows 83% aminoacid identity, 88% similarity with PKI55 aminoacid. At the nucleotide level is observed 93% sequence identity in the 5' regulatory region of PKI55. The direct derivation from a common ancestor, before the divergence between Old World Monkeys and Humans 25 Myr ago, appears as a more likely origin of the gene in the two lineages, rather than an horizontal transmission through infection between Macaca Fascicularis and Humans.

In the three PKI55 paralogs identified on chromosome 10, 15 e 20, identified by GenBank Accession Number respectively AL 356865, AC009432 and AL050403, corresponding to SEQ ID NO 3, 6, 8 of the sequence listing, and which are

encompassed by the present invention, the degree of sequence identity is highly significant and the regulatory regions also appear to be conserved: however their expression pattern and the modulating effect on single PKC isozymes strongly depends on the cellular system in which they are expressed.

The conservation of PKI55 sequence throughout the course of evolution, most likely, depends on a strong selective advantage conferred to the host by the gene product (32). The family of "paralogs" and orthologs represent an evolutionarily conserved system for the control of the activation of PKC isozymes in the various tissues.

The PKI55-PKC feedback loop, regulating PKC isozyme expression, may be a unique evolutionary adaptation system producing an "optimal running level" of activated PKC isozyme.

This may explain the selective advantage of the PKI55 sequence and the adaptive value of the PKI55-PKC system (42). Furthermore it indicates the clinical advantage that can be expected in vivo by the treatment of patients affected by diseases due to kinases' deregulation. The active part of PKI55 included in a compound suitable to the administration to Patients may be a very important "drug " whose preparation is one of the main aims of the present invention

The polynucleotides of the present invention also include, but are not limited to, polynucleotides that hybridize to the complement of the nucleotide sequence of SEQ ID NO 1 or to the complement of the sequences identified by accession n°: AL 356865, AC009432 and AL050403 under stringent hybridization conditions; a polynucleotide which is an allelic variant of any polynucleotide recited above; a polynucleotide which encodes a species homologue of any of the proteins recited above; or a polynucleotide that encodes a polypeptide comprising an additional specific domain or truncation of the polypeptide of SEQ ID NO 2, 4, 5, 7, 9, 11, 12, 13.

The invention also encompasses allelic variants of the disclosed polynucleotides; that is, naturally-occurring alternative forms of the isolated polynucleotide which also encode proteins which are identical, homologous or related to that encoded by the polynucleotides provided.

The sequences falling within the scope of the present invention are not limited to

the specific sequences herein described, but also include allelic variations thereof. Allelic variations can be routinely determined by comparing the sequence provided in SEQ ID NO 1 or to sequence identified with accession n°: AL 356865, AC009432 and AL050403. Furthermore, to accommodate codon variability, the invention includes nucleic acid molecules encoding for the same amino acid sequences, as do the specific ORFs disclosed herein. In other words, variation in the coding region of seqIDN1, or in the sequences AL356865, AC009432 and AL050403 or AB050412 (GenBank) which substitute one codon for another which encodes for the same amino acid are expressly contemplated by the present invention.

The present invention further provides isolated polypeptides encoded by the nucleic acid fragments of the present invention or by degenerate variants of the nucleic acid fragments of the present invention. By "degenerate variant" is intended nucleotide fragments which differ from a nucleic acid fragment of the present invention (e.g., an ORF) by nucleotide sequence, but due to the degeneracy of the genetic code, encode an identical polypeptide sequence.

The polynucleotides of the invention also provide polynucleotides including nucleotide sequences that are substantially equivalent to the polynucleotides recited above.

Polynucleotides according to the invention have at least about 55%, more typically at least about 70%, more typically at least about 80%, and even more typically at least about 90%, 95%, 98% (comprising the intermediate values) sequence identity to a polynucleotide recited above.

Methods and algorithms for obtaining such homologues polynucleotides are well known to those of skill in the art and can include, for example, methods for determining hybridization conditions, which can routinely isolate polynucleotides of the desired sequence identities or homology.

The invention also provides the complement of the polynucleotides comprised in the present invention.

Polynucleotides can be DNA (genomic, cDNA, amplified, or synthetic) or RNA.

Any of the polynucleotide according to the invention can be joined to any of a variety of other nucleotide sequences by well-established recombinant DNA

techniques (see Sambrook Jet al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY).

Useful nucleotide sequences for joining to polypeptides include an assortment of vectors, e.g., plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art.

Accordingly, the invention also provides vector including a polynucleotide of the invention and a host cell containing the polynucleotide. In general, the vector contains an origin of replication functional in at least one organism, convenient restriction endonuclease sites, and a selectable marker for the host cell. Vectors according to the invention include expression vectors, replication vectors, probe generation vectors, and sequencing vectors. A host cell according to the invention can be a prokaryotic or eukaryotic cell and can be a unicellular organism or part of a multicellular organism.

The recombinant constructs of the present invention comprise a vector, such as a plasmid or viral vector, into which a nucleic acid having the sequence of SEQ ID NO 1, or the sequence of GenBank Accession n°: AL 356865, AC009432 and AL050403 or their fragments thereof is inserted, in a forward or reverse orientation.

In the case of a vector comprising one of the ORFs of the present invention, the vector may further comprise regulatory sequences, including for example, a promoter, operably linked to the ORF. For vectors comprising the EMFs and UMFs of the present invention, the vector may further comprise a marker sequence or heterologous ORF operably linked to the EMF or UMF. Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention. The following vectors are provided by way of example:

The invention comprises both cloning and expression vectors comprising the nucleotide sequences disclosed.

The isolated polynucleotide of the invention may be operably linked to an expression control sequence such as T7, trc, lac for prokaryotic expression; GAL1, GAP or TEF1 for yeast expression; polyhedrin, MT or Ac5 for insect expression; CMV, SV40, EF-1α for mammalian expression, in order to produce the protein

recombinantly.

Eukaryotic or prokaryotic expression vectors are suitable for the expression of PKC modulator according to the present invention. Several expression systems either eukaryotic or prokaryotic are known in the art: for each of the recombinant expression system suitable expression control sequences have been identified and commonly used in the art.

General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185,537-566 (1990). As defined herein "operably linked" means that the isolated polynucleotide of the invention and an expression control sequence are situated within a vector or cell in such a way that the protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

Promoter regions may also be isolated from any desired gene sequence using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Appropriate expression vectors are for example commercially available: pTrcHis, pRSET, pPICZ, pTEF1, FastBac1, pcDNA.

Generally, a recombinant expression vector will include origins of replication and selectable markers allowing transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolic enzymes such as 3-phosphoglycerate kinase (PGK), a-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product. Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and

termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host.

The present invention further provides host cells genetically engineered to contain the polynucleotides of the invention, such as SEQ ID NO 1 or sequences corresponding to GenBank accession number: AL 356865, AC009432 and AL050403, or to AB050412, which express in a preferred embodiment the polypeptides according to the invention.

Such host cells are engineered to contain nucleic acids of the invention introduced into the host cell using known transformation, transfection or infection methods. The present invention still further provides host cells genetically engineered to express the polynucleotides of the invention, wherein such polynucleotides are in operative association with a regulatory sequence heterologous to the host cell, which drives expression of the polynucleotides in the cell.

The host cell can be a higher eukaryotic host cell, such as a mammalian cell, a lower eukaryotic host cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the recombinant construct into the host cell can be effected by calcium phosphate transfection, DEAE, dextran-mediated transfection, or electroporation (Davis, L. et al.. Basic Methods in Molecular Biology (1986). The host cells containing one of polynucleotides of the invention, can be used in conventional manners to produce the gene product encoded by the isolated fragment (in the case of an ORF) or can be used to produce a heterologous protein under the control of the EMF.

Any host/vector system can be used to express one or more of the nucleotides according to the present invention. These include, but are not limited to, eukaryotic hosts such as yeast, or mammalian cells, as well as prokaryotic host such as E. Coli. The most preferred cells are those which do not normally express the polypeptides of the present invention. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., in Molecular

Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, New York (1989), the disclosure of which is hereby incorporated by reference.

Various mammalian cell lines are employed to express the polypeptides of the present invention as recombinant proteins. Examples of mammalian cell lines comprise: Jurkat cell line and other cell lines capable expressing a compatible vector, for example, the HL-60, MCF-7, fibroblast cell lines, CHO, COS-1 or COS-7 cells HEK293 etc. Mammalian host cells include, for example, HeLa U937, CV1 and others.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences.

Recombinant polypeptides and proteins produced in bacterial culture are usually isolated by initial extraction from cell pellets, followed by one or more salting-out, aqueous ion exchange or size exclusion chromatography steps. Protein refolding steps can be used, when necessary, in completing configuration of the protein after bacterial expression. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. A number of types of cells may act as suitable host cells for expression of the protein.

Alternatively, it may be possible to produce the protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria.

Potentially suitable yeast strains include Schizosaccharomyces Pombe, Saccharomyces Cerevisiae, Pichia Pastoris, Pichia Methanolica, Candida or any yeast strain capable of expressing heterologous proteins.

Potentially suitable bacterial strains include Escherichia coli, Bacillus subtilis, Salmonella typhimurium, or any bacterial strain capable of expressing heterologous proteins. If the protein is made in yeast or bacteria, it may be necessary to modify the protein produced therein, for example by phosphorylation or glycosylation of the appropriate sites, in order to obtain the functional protein.

Such covalent attachments may be accomplished using known chemical or enzymatic methods. In order to optimize expression in bacteria, it may also be necessary to modify the nucleotide sequence according to the bacterial preferred codon codon usage.

In another embodiment of the present invention, cells and tissues may be engineered to express an endogenous gene comprising the polynucleotides of the invention under the control of inducible regulatory elements, in which case the regulatory sequences of the endogenous gene may be replaced by homologous recombination. As described herein, gene targeting can be used to replace a gene's existing regulatory region with a regulatory sequence isolated from a different gene or a novel regulatory sequence synthesized by genetic engineering methods.

Polypeptides according to present invention are produced chemically, naturally from the cells, or recombinantly according to methods known in the art.

According to a further embodiment, the invention also provides for polypeptides or peptides and polynucleotides for pharmaceutical use. Protein compositions of the present invention may further comprise an acceptable carrier, such as a pharmaceutically acceptable carriers known in the art, liposomes being comprised.

The invention also relates to methods for producing a polypeptide comprising growing a culture of the cells of the invention in a suitable culture medium, and purifying the protein from the culture. For example, the methods of the invention include a process for producing a polypeptide in which a host cell containing a suitable expression vector that includes a polynucleotide of the invention is cultured under conditions that allow expression of the encoded polypeptide. The polypeptide can be recovered from the culture, conveniently from the culture medium, and further purified. Preferred embodiments include those in which the protein produced by such process is a full length or mature form of the protein.

Preferred nucleic acid fragments of the present invention are the ORFs that encode proteins. A variety of methodologies known in the art can be utilized to obtain any one of the isolated polypeptides or proteins of the present invention. At the simplest level, the amino acid sequence can be synthesized using commercially available peptide synthesizers. This is particularly useful in

producing small peptides and fragments of larger polypeptides.

Fragments are useful, for example, in generating antibodies against the native polypeptide. In an alternative method, the polypeptide or protein is purified from bacterial cells which naturally produce the polypeptide or protein. One skilled in the art can readily follow known methods.

The compositions of the present invention include isolated polynucleotides, including recombinant DNA molecules, cloned genes or degenerate variants thereof, especially naturally occurring variants such as allelic variants, novel isolated polypeptides, and antibodies that specifically recognize one or more epitopes present on such polypeptides. The polypeptide can be expressed as a product of a transgenic animal, modified in order to contain cells containing a nucleotidic sequence encoding for the protein.

BIOLOGICAL ACTIVITY AND USES

The polynucleotides and proteins of the present invention are expected to exhibit one or more of the uses or biological activities (including those associated with assays cited herein) identified below. Uses or activities described for proteins of the present invention may be provided by administration or use of such proteins or by administration or use of polynucleotides encoding such proteins (such as, for example, in gene therapies or vectors suitable for introduction of DNA).

RESEARCH USES AND UTILITIES

The polynucleotides provided by the present invention can be used by the research community for various purposes. The polynucleotides can be used to express recombinant protein for analysis, characterization or therapeutic use; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides; for selecting and making

oligomers for attachment to a "gene chip" or other support, including for examination of expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response. Where the polynucleotide encodes a protein which binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the polynucleotide can also be used in interaction trap assays (such as, for example, that described in Gyuris et al., Cell 75 :791-803 (1993)) to identify polynucleotides encoding the other protein with which binding occurs or to identify inhibitors of the binding interaction.

The proteins provided by the present invention can similarly be used in assay to determine biological activity, including in a panel of multiple proteins for high-throughput screening; to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands. Where the protein binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

Due to the crucial activity of PKC and to the novel mechanism elucidated for this family of modulators, the informations disclosed herein open up new applications

in the diagnostic as well as in the therapeutic field. Moreover the present invention further provides an essential research tool for the setting up of in vitro and in vivo models in the laboratory practice.

The invention is not limited to the sequences disclosed but extends to all the related sequences isolated by the use of sequences IDN1-12.

In the therapeutic field the compounds according to the present invention are of particular interest whenever a dis-regulation or a defect in Protein Kinase C regulation is observed. Generally speaking this mechanism is extremely important whenever a defect/disregulation in the signal transduction machinery may be pinpointed: these include, non-exhaustively: defects in cell proliferation such as autoimmune or allergic diseases, oncologic and neurologic diseases, in particular related to iskaemic damage. In the diagnostic field the nucleic acids and polypeptides according to this invention are of interest in the diagnosis of inherited or non inherited diseases such as those mentioned above.

A protein of the present invention (from whatever source derived, including without limitation from recombinant and non-recombinant sources) may be administered to a patient in need, by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s) at doses to treat or ameliorate a variety of disorders. Preferred pharmaceutical carriers are represented by liposomes. Such compositions may also contain (in addition to protein and a carrier) diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration.

Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, P A, latest edition. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms, e.g., treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone.

When applied to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous. intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Administration of protein of the present invention used in the pharrnaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, topical application or cutaneous, subcutaneous, intraperitoneal, parenteral or intravenous injection.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into arthritic joints or in fibrotic tissue, often in a depot or sustained release formulation. In order to prevent the scarring process frequently occurring as complication of glaucoma surgery, the compounds may be administered topically, for example, as eye drops. Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a specific antibody, targeting, for example, arthritic or fibrotic tissue. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. These pharmaceutical compositions may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen. When a therapeutically effective amount of protein of the present invention is administered orally, protein of the present invention will be in the form of a tablet, capsule, powder, solution or elixir. When administered in

tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder preferably contain from about 5 to 95% protein of the present invention, and more preferably from about 25 to 90% protein of the present invention. When administered in liquid mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition preferably contains from about 0.5 to 90% by weight of protein of the present invention, and more preferably from about 1 to 50% protein of the present invention.

When a therapeutically effective amount of protein of the present invention is administered by intravenous, cutaneous or subcutaneous injection, protein of the present invention will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to protein of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and

the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration. For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit

may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides. In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection.

Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Liposomes and emulsions are well known examples of delivery vehicles or

carriers. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Several sustained-release materials have been established and are well known by those skilled in the art.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Many of the proteinase inhibiting compounds of the invention may be provided as salts with pharmaceutically compatib1e counterions. Such pharmaceutically acceptable base addition salts are those salts which retain the biological effectiveness and properties of the free acids and which are obtained by reaction with inorganic or organic bases such as sodium hydroxide, magnesium hydroxide, ammonia, trialkylamine, dialkylamine, monoalkylamine, dibasic amino acids, sodium acetate, potassium benzoate, triethanol amine and the like.

The amount of protein of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments, which the patient has undergone. Ultimately, the attending, physician will decide the amount of protein of the present invention with which to treat each individual patient.

The various pharmaceutical compositions used to practice the method of the present invention should contain about 0.01 mg to about 100 mg, preferably about 0.1 mg to about 10 mg, more preferably about 0.1 mg to about 1 mg of protein of the present invention per kg body weight.

For compositions of the present invention which are useful for bone, cartilage, tendon or ligament regeneration, the therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of

bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than a protein of the invention which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering the protein-containing composition to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyglycolic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are non-biodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The therapeutic compositions are also presently valuable for veterinary applications. Particularly domestic animals and thoroughbred horses, in addition to humans, are desired patients for such treatment with proteins of the present invention. The dosage regimen of a protein-containing pharmaceutical composition to be used in tissue regeneration will be determined by the attending physician considering various factors which modify the action of the proteins, e.g., amount of

tissue weight desired to be formed, the site of damage, the condition of the damaged tissue, the size of a wound, type of damaged tissue (e.g., bone), the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and with inclusion of other proteins in the pharmaceutical composition.

Polynucleotides of the present invention can also be used for gene therapy. Such polynucleotides can be introduced either in vivo or ex vivo into cells for expression in a mammalian subject. Polynucleotides of the invention may also be administered by other known methods for introduction of nucleic acid into a cell or organism (including, without limitation, in the form of viral vectors or naked DNA). Cells may also be cultured ex vivo in the presence, proteins of the present invention in order to proliferate or to produce a desired effect on or activity in such cells. Treated cells can then be introduced in vivo for therapeutic purposes.

ANTIBODIES

Another aspect of the invention is an antibody that specifically binds the polypeptides of the invention in all their embodiments. Such antibodies can be either monoclonal or polyclonal antibodies, as well fragments thereof and humanized forms or fully human forms, such as those produced in transgenic animals. The invention further provides a hybridoma that produces an antibody according to the invention. Antibodies of the invention are useful for detection and/or purification ofthe polypeptides of the invention.

DIAGNOSTIC ASSAYS AND KITS

The present invention further provides methods to identify the presence or expression of one of the ORFs of the present invention, or homolog thereof, in a test sample, using a nucleic acid probe or antibodies of the present invention.

In general, methods for detecting a polynucleotide of the invention can comprise contacting a sample with a compound that binds to and forms a complex with the polynucleotide for a period sufficient to form the complex, and detecting the complex, so that if a complex is detected, a polynucleotide of the invention is detected in the sample. Such methods can also comprise contacting a sample under stringent hybridization conditions with nucleic acid primers that anneal to a

polynucleotide of the invention under such conditions, and amplifying annealed polynucleotides, so that if a polynucleotide is amplified, a polynucleotide ofthe invention is detected in the sample.

In general, methods for detecting a polypeptide of the invention can comprise contacting a sample with a compound that binds to and forms a complex with the polypeptide for a period sufficient to form the complex, and detecting the complex, so that if a complex is detected, a polypeptide of the invention is detected in the sample.

In detail, such methods comprise incubating a test sample with one or more of the antibodies or one or more of nucleic acid probes of the present invention and assaying for binding of the nucleic acid probes or antibodies to components within the test sample.

Conditions for incubating a nucleic acid probe or antibody with a test sample may vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the nucleic acid probe or antibody used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or imrnunological assay formats can readily be adapted to employ the nucleic acid probes or antibodies of the present invention. Examples of such assays can be found in Chard, T., An Introduction to Radioimmunoassay and Related Techniques, Elsevier Science Publishers, Amsterdarn, The Netherlands (1986); Bullock, G.R. et al., Techniques in Immunocytochemistry, Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., Practice and Theory of immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdarn, The Netherlands (1985). The test sarnples of the present invention include cells, protein or membrane extracts of cells, or biological fluids such as sputum, blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention. Specifically, the invention provides a compartment kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the probes or antibodies of the present invention; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound probe or antibody.

In detail, a compartment kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic, or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sarnple, a container which contains the antibodies used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or probe. Types of detection reagents include labeled nucleic acid probes, labeled secondary antibodies, or in the alternative, if the primary antibody is labeled, the enzymatic, or antibody binding reagents which are capable of reacting with the labeled antibody. One skilled in the art will readily recognize that the disclosed probes and antibodies of the present invention can be readily incorporated into one of the established kit forrnats which are well known in the art.

DRUG SCREENING ASSAYS

Using the isolated proteins and polynucleotides disclosed above, the present invention further provides methods of obtaining and identifying agents which bind either to the polypeptides of the invention, or to a specific domain of said polypeptide, or to the PKC isoforms, or to identify the PKC domain involved in binding the polypeptides of the invention.

In detail, said method comprises the steps of: (a) contacting an agent with an isolated protein encoded by an ORF of the present invention, or nucleic acid of the invention; and (b) determining whether the agent binds to said protein or said

nucleic acid.

In general, therefore, such methods for identifying compounds that bind to a polynucleotide of the invention can comprise contacting a compound with a polynucleotide of the invention for a time sufficient to form a polynucleotide/compound complex, and detecting the complex, so that if a polynucleotide/compound complex is detected, a compound that binds to a polynucleotide of the invention is identified.

Likewise, in general, therefore, such methods for identifying compounds that bind to a polypeptide of the invention can comprise contacting a compound with a polypeptide of the invention for a time sufficient to form polypeptide/compound complex, and detecting the complex, so that if a polypeptide/compound complex is detected, a compound that binds to a polynucleotide of the invention is identified.

Methods for identifying compounds that bind to a polypeptide of the invention can also comprise contacting a compound with a polypeptide of the invention in a cell for a time sufficient to form a polypeptide/compound complex, wherein the complex drives expression of a receptor gene sequerice in the cell, and detecting the complex by detecting reporter gene sequence expression, so that if a polypeptide/compound complex is detected, a compound that binds a polypeptide of the invention is identified.

Compounds identified via such methods can include compounds which modulate the activity of a polypeptide of the invention (that is, increase or decrease its activity, relative to activity observed in the absence of the compound). Alternatively, compounds identified via such methods can include compounds which modulate the expression of a polynucleotide of the invention (that is, increase or decrease expression relative to expression levels observed in the absence of the compound). Compounds, such as compounds identified via the methods of the invention, can be tested using standard assays well known to those of skill in the art for their ability to modulate activity/expression.

The agents screened in the above assay can be, but are not limited to, peptides, carbohydrates, vitamin derivatives, or other pharmaceutical agents. The agents can be selected and screened at random or rationally selected or designed using protein modeling techniques.

For random screening, agents such as peptides, carbohydrates, pharmaceutical agents and the like are selected at random and are assayed for their ability to bind to the protein encoded by the ORF of the present invention. Alternatively, agents may be rationally selected or designed. As used herein, an agent is said to be "rationally selected or designed" when the agent is chosen based on the configuration of the particular protein. For example, one skilled in the art can readily adapt currently available procedures to generate peptides, pharmaceutical agents and the like capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby et al. , Application of Synthetic Peptides: Antisense Peptides," In Synthetic Peptides, A User 's Guide, W .H. Freeman, NY (1992), pp. 289-307, and Kaspczak et al., Biochemistry 28:9230-8 (1989), or pharmaceutical agents, or the like.

In addition to the foregoing, one class of agents of the present invention, as broadly described, can be used to control gene expression through binding to one of the ORFs of the present invention. As described above, such agents can be randomly screened or rationally designed/selected. Targeting the ORF allows a skilled artisan to design sequence specific or element specific agents, modulating the expression of either a single ORF or multiple ORFs which rely on the same transcriptional regulatory control region for expression control. One class of DNA binding agents are agents which contain base residues which hybridize or form a triple helix formation by binding to DNA or RNA. Such agents can be based on the classic phosphodiester, ribonucleic acid backbone, or can be a variety of sulfhydryl or polymeric derivatives which have base attachment capacity.

Agents suitable for use in these methods usually contain 20 to 40 bases and are designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res. 6:3073 (1979); Cooney et al., Science 241 :456 (1988); and Dervan et a/.. Science 251:1360 (1991) or to the mRNA itself (antisense - Okano, J: Neurochem. 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Express;on, CRC Press, Boca Raton, FL (1988)). Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in

model systems.

Information contained in the sequences of the present invention is necessary for the design of an antisense or triple helix oligonucleotide and other DNA binding agents. Agents which bind to a protein encoded by one of the ORFs of the present invention can be used as a diagnostic agent, in the control of bacterial infection by modulating the activity of the protein encoded by the ORF. Agents which bind to a protein encoded by one of the ORFs of the present invention can be formulated using known techniques to generate a pharmaceutical composition.

USE OF NUCLEIC ACIDS AS PROBES

Another aspect of the invention is to provide for polypeptide-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences for the detection of altered levels of PKI55 or their derivatives mutant , allelic variants, paralogs or orthologs expression. This specificity of assays may be also used as screening methods to detect altered levels of PKC expression.

The hybridization probes of the present invention may be derived from the nucleotide sequence of the SEQ ID NO:1 or from sequences corresponding to GenBank Accession number AB050412, AL356865, AC 009432, AL050403. Because the corresponding gene is only expressed in a limited number of tissues a hybridization probe derived from such sequences can be used as an indicator of the presence of RNA of cell type of such a tissue in a sample.

Any suitable hybridization technique can be employed, such as, for example, in situ hybridization.PCR as described US Patent Nos 4,683, 195 and 4,965, 188 provides additional uses for oligonucleotides based upon the nucleotide sequences. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both. The probe will comprise a discrete nucleotide sequence for the detection of identical sequences or a degenerate pool of possible sequences for identification of closely related genomic sequences.

Other means for producing specific hybridization probes for nucleic acids include the cloning of nucleic acid sequences into vectors for the production of rnRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate

radioactively labeled nucleotides. The nucleotide sequences rnay be used to construct hybridization probes for mapping their respective genornic sequences. The nucleotide sequence provided herein may be mapped to a chromosome or specific regions of a chromosome using well known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries or flow-sorted chromosomal preparations specific to known chromosomes, and the like. The technique of fluorescent in situ hybridization of chromosome spreads has been described, among other places, in Venna et al (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY.

Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265: 1981 f). Correlation between the location of a nucleic acid on a physical chromosomal map and a specific disease (or predisposition to a specific disease) may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier or affected individuals. The nucleotide sequence may be used to produce purified polypeptides using well known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Goeddel (1990) Gene Expression Technology, Methods and Enzymology, Vol 185; Academic Press, San Diego. Polypeptides may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which a particular polypeptide nucleotide sequence was isolated or from a different species. Advantages of producing polypeptides by recombinant DNA technology include obtaining adequate arnounts of the protein for purification and the availability of simplified purification procedures.

The present invention will be illustrated by the following examples and Figures which are not to be considered as limiting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Transcription-translation analysis

DNA control (lane a), pBluescript including the cDNA of IMAGE clone (lane b), cpBluescript alone (lane c). Fractions of each reaction (1/3) were separated by 12% SDS-P AGE. Molecular size markers are indicated on the left. A single 6.5 kDa band was detectable in lane b.

Figure2. Selected genomic structure of KI55 gene

cDNA IMAGE clone m 38900 included between 87205-88756bp (1551bp) of BAC clone ACO08123 is shown. The 533bp LTR32/ERVL region (italic) from 88270 to 88803bp of ACO08123 includes the KI55 coding sequence is indicated between the two arrows; amminoacids of the coded protein are in capital letters. The promoter at position 88431 (-102 bp from the first AUG of the coding sequence), the TATA box at position 88406 (-25 bp from the promoter), an AP-I consensus sequence at 87379bp (-1052bp from the promoter) and a second one at 88456 (+25 bp from the promoter) are indicated above the sequence. Consensus sequences for common transcription factors in the enhancer region are underlined. Double underline indicates overlapping consensus sequences.

Figure 3. Eadie-Hofstee plot

A) Eadie-Hofstee plot of PKCα isozyme inhibition by PKI55. K_d was calculated from the point of intersection on the abscissa. (14 μM)

B) Eadie-Hofstee plot of PKC δ isozyme inhibition by PKI55. K_d was calculated from the point of intersection on the abscissa (13 μM).

Figure 4 Nuclear Magnetic Resonance (NMR)

Portion of the 1H-NMR spectrum of PKI55 in aqueous solution (20% D2O, protein concentration 1 mg/ml, pH 7, 25 °C). The spectrum was acquired on a Varian Unity Inova 400 spectrometer using an acquisition time of 2 s and 512 transients.

Figure 5. Western Blot analysis

sPKI55 and human recombinant PKCα were run together (sample a) and stained with anti-PKCα antibody (lane a') and with anti-PKI55 (lane a"): two bands at the specific MW are visible. When Ca^{2+}, PS and DAG were added to sPKI55 plus PKC alpha (sample b), a single macromolecular complex 87 kDa was visible and stained using anti-PKCalpha antibody (lane b') and with anti-PKI55 antibody (lane b"). Molecular weights are shown on the left.

Figure 6. Coimmunoprecipitation experiments.

87 kDa band is indicated (arrow) in lysates from: untreated PBMC (lane 1), PHA-activated PBMC (lane 2), PMA-activated PBMC (lane 3) immunoprecipitated by: A) anti-PKCα antibody and stained with anti-PKI55 antibody, B) anti-PKI55 antibody and stained with anti-PKCα antibody. Molecular weight markers are shown on the right.

Figure 7. Effect of PKI55 on the degradation of PKC by calpain.

Human erythrocyte calpain was incubated as described in Experimental Procedures with PKC in the absence (A) and presence (B and C) of PKI55. In C, PS and DAG were also present in the incubation mixture. At the indicated time, PKC activity was assayed in the presence of 1mM EDTA (filled circles) and PS and DAG (filled triangles).

Figure 8: PKI55-PKC feedback.

PKI55 synthesis was assayed in lysates of PBMC cultured for 1h.

a: unstimulated PBMC (lane 1), PHA-activated (lane 2), OKT3-activated (lane 3), PHA-activated plus H7 (lane 4), OKT3-activated plus H7 (lane 5), PHA-activated plus cycloheximide (lane 6), OKT3-activated plus cycloheximide (lane 7). PMA-activated (lane 8), PMA-activated plus H7 (lane 9), PMA-activated plus cycloheximide (lane 10). PKC α, PKI55 and molecular weight markers (kDa) control lanes are on the left.

b: cell lysates of PMA-stimulated PBMC were studied at 0.5h, 1h, 24h, 48h, 72h, quantifying activated PKCα (80 kDa), degraded PKCα form (PKM) (45 kDa), PKI55-PKCα complex (87 kDa), PKI55 (6.5 kDa). Results are expressed in Densitometric Unit / mm2. Molecular weights (kDa) are on the right.

Figure 9. Similarities of the query PKI55 amminoacid sequence

A)Refined Alignment, B) Parameters 1

Figure 10. Physical and restriction map of the pRc/RSV restriction vector

Terms used in Figure 10:

RSV promoter: bases 209-605

Polylinker: bases 606-705

BGH Poly A signal: bases 706-932

F1 origin: bases 988-1510

SV40 promotor: bases 1572-1897

SV40 origin of replication: bases 1766-1851

Neomycin gene: bases 1903-2697

SV40 Poly A signal : bases 2701-2910

ColE1 origin: bases 3233-3756

Ampicillin resistance gene: bases 4240-5100

pUC backbone, origin and ß-lactamase gene: begins at base 3009

The unique sites in the polylinker are: Hind III, Spe I, BstX I, Not Iand Xba I

Figure 11. Cell proliferation estimated by vitality test with count with Tripan Blue

Count with Tripan Blue to estimate vitality of cells transfected with:

pRc/RSV plasmid inserted with the gene in correct direction (RSV-senso, -▲-)

control plasmid (RSV, -■-)

the plasmid carrying the gene in the antisense (RSV-anti, -X-)

As a control, count with Tripan Blue of cell not transfected (Jurkat, -♦-)

On top the table shows the values obtained at indicated times.

Figure 12. Acetilcoline release from cerebral cortex stimulated at 10Hz

Acetilcoline release after cortex stimulation at 10Hz during time cerebral cortex Vs control (100%) in the presence of 50 µM of the G16 peptide in presence of O_2 (GW16, -*-), 50 µM of the G16 peptide in presence of N_2 (GW16, -▲-), 50 µM of the F20 peptide in presence of N_2 (FE20, -♦-), control iskemia (-■-).

Figure 13. Localization of PKI55-FITC in Resting and Activated Lymphocytes.

Permeabilized (A, B and C) and nonpermeabilized PBMC (D, E and F) from a normal donor were studied as described in Experimental Procedure. Propidium iodide (red fluorescence) was used to mark the nucleus. Fluorescinated sPKI55 (green fluorescence) was added to the culture medium. In the permeabilized cells both B (PMA activated) and C (Ca^{2+}ionofore-activated) show green fluorescence. In the nonpermeabilized cells, green fluorescence was obtained only in F (Ca^{2+}ionofore-activated), whereas in E (PMA-activated) no green fluorescence was present. Unstimulated PBMC (A and D) also showed no green fluorescence.

Figure 14. Dot blot analysis with anti-PKI55 antibody.

Top line: increasing amounts of sPKI55, as indicated, were tested against rabbit polyclonal anti-PKI55 serum (1:100). Second line: concentrated supernatants (10

ug) from unstimulated PBMC (a), PHA-activated PBMC (b), OKT3-activated PBMC (c). Third line : concentrated cell lysates from unstimulated PBMC (a), PHA-activated PBMC (b), OKT3-activated PBMC (c).

EXPERIMENTAL PART

MATERIALS AND METHODS

Reagents

Human recombinant PKC isozymes were purchased from Calbiochem (Inalco), polyclonal anti-PKCα and δ were from Santa Cruz and PHA from Burroughs Wellcome. All other reagents were reagent grade. Calpain was purified from human erythrocytes according to Michetti (11).

Cell Cultures

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by Hystopaque gradient centrifugation. Cells were suspended at $1x10^6$ cells/m1 in Iscove Dulbecc modified medium (Gibco), supplemented with 10% heat-inactivated Fetal Calf Serum, 50U/ml Penicillin, 50μg/ml streptomycin and cultured in Heraeus at 37°C in a 10% CO_2 humidified atmosphere for 1h. Phytohaemagglutinin (PHA) was administered at 1μg/ml, phorbol-myristate-acetate (PMA) at 1nM and monoclonal antibody anti-CD3 (OKT3, 10 μg/ml) was pre-coated in 0.05 M carbonate-bicarbonate buffer pH 9.6 at 4°C overnight in 96-well round bottom plates (Nunc). PKC inhibitor [1-(5-isoquinolinylsulphonyl)-2] methylpiperazine (iso-H7, Sigma) was used at the specific PKC inhibiting concentration: 25 μM (12). Cycloheximide was administered at 10μg/ml.

The Jurkat lymphoblastoid cell line derived from human leukemia T cells were grown in Iscove's Modified Dulbecco Minimum Essential Medium (DMEM Gibco)supplemented with 10% Fetal Calf serum and antibiotics in Heraus incubator at 37°C with modified 10% CO_2 atmosphere.

Supernatant Concentrates

Cells were cultured for 1h in Heraeus and the media were collected. Centricon Biomax-30 (Millipore) were used to purify the medium from high molecular weight proteins. The filtrate was dialysed with 3500 Da cut-off tubes. Samples were liophilized and resuspended in a small volume of water and protein concentration was detected by 280nm spectrophotometric analysis.

Protein separation was performed using fast protein liquid chromatography (FPLC). Superdex 75 HR 10/30 (Pharmacia) equilibrated with sodium borate 50mM pH 7,5 containing 50mM NaCl was used. Gel filtration was carried out at 1ml/min and 400µl fractions were collected.

Fluorescent in situ Hybridization (FISH) analysis

A CEPH YAC genome library obtained from the YAC Screening Centre of DIBIT (San Raffaele Scientific Institute, Milan) was screened using PCR. Positive YAC clones were transferred from agarose gel (PFGE) to GeneScreen membrane filters (Dupont) and hybridized with labelled KI55 by standard procedures (13). A unique single hybridization signal was obtained in 797A11 DNA that was labelled by nick translation with biotin-16-dUTP and used to hybridize a metaphasic chromosome preparation of human T-lymphocytes. The detection was carried out by FITC-conjugate avidin.

NMR study

The relationship between the structural conformation of PKI55 and its biological activity was studied by mono-dimensional IH-NMR, bidimensional 1H-ROESY NMR spectroscopy and the circular dicroism (CD) technique.

The NMR spectrum was acquired on a Varian Unity Inova 400 spectrometer using an acquisition time of 2 s and 512 transients.

Fig. 4 displays the low field region of the 1H-NMR spectrum of the protein. This narrow region comprises the rather overlapping resonance of the peptidic NH's (~ 8-9 ppm) and side chain NH's or aromatic CH's (~ 6.5-7.5 ppm)

In Vitro Coupled Transcription-Translation

An aliquot of 1 µg of the circular plasmid pBluescript containing the 1530 bp from IMAGE clone was transcribed and translated using the TNT® T7-coupled reticulocyte lysate system (Promega), according to the manufacturer's recommendations. DNA and pBluescript controls were included. The reaction was performed at 37°C for 90 min in the presence of [^{35}S]methionine. SDS-PAGE was carried out under reducing condition in a Mini-Protean electrophoresis unit (Bio-Rad) according to Laemmli (14). The gel was then fixed for 20 min in a solution of 25% ethanol and 10% acetic acid, amplified with the Amplify solution (Amersham Pharmacia), dried under vacuum and exposed for 24 h at -80°C to Hyperfilm-MP

x-ray film.

Recombinant PKI55 Preparation

KI55 coding sequence was cloned in frame in pTrcHis-A (Invitrogen). The fusion protein was purified by a nickel matrix (Probond, Invitrogen) and re-covered by imidazole elution. After enterokinase digestion, the recombinant PKI55 (rPKI55) was recovered by gel filtration on a Superdex 75HR column (Pharmacia).

Automated Protein Synthesis and Purification of synthetic PKI55 (sPKI55)

Peptide synthesis was carried out on a 0.1mmol scale of synthetic PKI55 (sPKI55) (Applied Biosystems, model 431A) using Na-9-fluorenylmethoxycarbonyl (Fmoc) amino acids and a preloaded Wang resin (Calbiochem-Novabiochem AG Loufelfinger, Switzerland). Couplings were performed in dimethylformamide using N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The following side chain-protecting groups were used: tert-butyl for Asp, Ser, Glu, Thr and Tyr; trityl for Asn, and Gln, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg and butyloxycarbonyl for Lys. Assembled peptides were exposed and cleaved from the polymer support by treatment with a cleavage mixture (10ml trifluoracetic acid 0.5ml thioanisole, 0.25ml ethanedithiol, 0.75g crystalline phenol, 0.5ml H_2O) for several hours. Cleaved peptides were then concentrated on a rotary evaporator to a volume of approximately 2 ml. Peptides are precipitated by the addition of 50ml of cold Ethyl Alcohol Absolute, collected by filtration through a fine porosity fritter glass funnel, dried under a high vacuum and dissolved in an aqueous solvent for lyophilization. Peptides were then lyophilized and dissolved in water 3 to 4 times and purified by reversed-phase HPLC on a Vydac C18 preparative column (Vydac, Hesperia, CA). All peptides were analyzed by matrix-associated laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry (Perseptive Biosystems).

PKC isolation and Assay

Cells were suspended at 20-50x10^6 cells/ml in 0.25M Sucrose, 10mM Hepes pH 7.5, 5mM EDTA, 10mM 2-Mercaptoethanol, 2mM Phenyl-Methyl-Sulphonylfluoride and 0.01% Leupeptin. The lysate was sonicated and centrifuged at 100,000 rpm for 10min to separate the cytosolic fraction (supernatant) from the particulate fraction (pellet). The latter was resuspended in the same buffer added with 0,1%

Triton-X100. Cytosolic and particulate PKC were eluted with a linear gradient 0-0.4M NaCl on a DEAE-cellulose column. The PKC activity was assayed in a mixture containing 50mM sodium borate pH7.5, 5μM ^{32}P-ATP (6000 Ci mmol-1 ATP), 5mM $MgCl_2$, 100μg of type III-S Histone, 10μg of phosphatidylserine, 0.2μg of dioleoylglycerol, 0.5 mM Ca^{2+} and the appropriate amount of enzyme source as described (15).

Kinetic Analysis

The activity of the specific recombinant isozymes was assayed by measuring the initial rate of phosphate incorporation from ^{32}P-ATP into saturating amounts of histone (32μM) as described (16-17).

The kinetics analysis was carried out using the equation:

inhibition = - K_d Inhibition derived from the Eadie-Hofstee equation

[PKI55]+max inhibition

K_d was determined from the point of intersection on the abscissa corresponding to Max inhibition

K_d

The activity of the following synthetic peptides derived from the PKI55 was measured by dose dependency:

1-4: MLYK

5-10: LHDVCR

1-10: MLYKLHDVCR

1-16: MLYKLHDVCRQLWFSC

1-26: MLYKLHDVCRQLWFSCPACHHRAMRI

A measurable value of % inhibition was detected in the concentration range 200nM-100μM of the above peptides.

Kd and IC-50 correspond to the inhibitor concentration that causes 50% inhibition and measure the affinity of the Inhibitor-Enzyme reaction.

Kd was calculated from the following equation inhibition = - K_d Inhibition (derived from the Eadie-Hofstee equation)/[PKI55]+max inhibition

K_d was determined from the point of intersection on the abscissa corresponding to Max inhibition.

IC50 is the peptide concentration value that in a dose dependent titration gives

50% inhibition of the activated PKC isozyme.

Antibody Preparation and Characterisation

Male Sprague Dawley rats and two adult New Zealand white rabbits were immunized with BSA-conjugated purified sPKI55 according to the PIERCE procedure of the Inject Immunogen EDC Conjugation kit as described (18). Polyclonal anti-PKI55 immune serum from rat or rabbit were tested on increasing doses of sPKI55 transferred to PVDF (0.2μ BioRad) using ECL Western blotting detection reagents (Amersham). Regression analysis of dot-blot densitometric units against known doses of sPKI55 yielded the estimates of PKI55 in the test-samples. The specific anti-PKI55 IgG were produced in rabbits and characterized following the immunochemical standard procedures to test the specific PKI55.antigen detection in biological samples. The preimmune rabbit serum and the rabbit IgG from the preimmune serum were used to detect no specific immunoreaction against PKI55. These experiments are generally considered exhaustive to use immune IgG to specifically recognize the antigen PKI55 as reported (19).

Coimmunoprecipitation

Cells were lysed on ice with 300ul of lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.5 Na Deoxycholic acid, 1% SDS, 10μg/ml leupeptin, 1mM PMSF, 10μg/ml aprotinin) and the suspensions were centrifuged at 400rpm for 10 min. Supernatants were preadsorbed with Protein G/A-Sepharose beads (Amersham Pharmacia) for at least 3 hours at 2-8°C on a rocking platform. Aliquots of supernatants were incubated for 1 hour at 4°C with specific antibodies: anti-PKCα or anti-PKI55 and the incubation were continued overnight with the addition of Protein A-Sepharose.

Immunocomplexes were pelletted by centrifugation at 2500rpm for 20 sec., washed two times with lysis buffer, two times with washing buffer 2 (50mM Tris pH 7.5, 500 mM NaCl. 0.1%NP-40. 0.05% sodium deoxycholate) and once with washing buffer 3 (50 mM Tris pH 7.5, 0.1% NP-40. 0.05% sodium deoxycholate). The immunoprecipitated protein complexes were eluted from agarose beads. fractionated by 10% SDS-PAGE and probed by Western blot with anti-PKI55 and anti-PKCα antibodies separately.

For the cytosolic fraction assay 50-100μg of partially purified PKC were added to the SDS gel. Primary antibodies against PKC isozymes were obtained from Santa Cruz Biotechnology.

Immunofluorescence Confocal Microscopy

PBMC from healthy donor were fixed and treated or not with Triton/paraformaldehyde method as described (Higgins and Sharp, 1988). Non-specific protein binding to cells was blocked by 30min incubation with 5% (v/v) fetal calf serum diluted in PBS. Cells were then treated with 2μg/ml FITC-PKI55 for 1 h. Staining of chromatin was carried out by incubating fixed cells with 2μg/ml propidium iodide for 5min. Cells were mounted on coverslips with FluoroGard anti-fade reagent (BioRad Laboratories) before analysis. Image of the samples were collected the same day by confocal microscopy using a BioRad MRC1024 instrument (krypton/argon laser) on a Nikon Diaphot 200, using a planapochromat x 60 oil-immersion objective with numerical aperture 1,40. The excitation/emission wavelength were 488/522 nm from fluorescein-labelled antibodies and 488/605 nm for propidium iodide-stained chromatin. When indicated, cells ($1x10x^6$ /ml) were treated with PMA (100ng/ml) or with Ca^{2+} ionofore (A23187) (1μM) incubated for10 min at 37°C and fixed as described above.

Bioinformatic Study

In a first analysis we carried out a similarity search of the available sequences databases with an aminoacid BLAST query sequence of PKI55 (19).

In a second analysis, a pairwise BLAST nucleotide sequence was performed with a nucleotide query sequence of the cDNACloneDKFZp434H1419 that is included in 2q35 BAC Clone RPCI11-1064L18 from 86,123 to 88,735 bp and includes, therefore, thePKI55 coding sequence and regulating region.

Southern and Northern Blotting

Genomic DNA was isolated and processed from cells as described (9). Total RNA was isolated from cultured cells as described (10). The coding portion of KI-55, obtained by PCR, was purified and labelled with α^{32}P-dCTP by random priming. The hybridizations were performed overnight at 42°C in 50% formamide, 2XSSC, 1% SDS, 0.5mg/ml denatured salmon sperm DNA. The membranes were washed for 10 min at room temperature in 2X SSC, 1% SDS; then washed twice for 15 min

at 0.2 X SSC, 1% SDS and exposed to Hyperfilm-MP (Amersham) for 3-5 days.

Western Blot

A partially denatured SDS-polyacrilamide gel was used to adjust the conditions to separate the proteins (20). Samples, containing equal amounts of proteins. were diluted with loading buffer without SDS and 2-mercaptoethanol (187.5mM Tris-HCl pH6.8. 30% glycerol, 0.003% bromophenol blue) and subjected to a 10% polyacrilamide gel containing a very low amount of SDS (0.01%). The protein transferring to the PVDF membrane (Bio-Rad, 0.2μ) was by electroblotting. Separated proteins were probed with anti-PKCα or anti-PKI55 antibodies. Protein bands were visualized using an enhanced chemiluminescence method (Amersham).

Assay of Calpain Activity and PKC Digestion

Calpain activity was assayed using human denatured globin as a substrate, in the presence of 1mM Ca^{2+} as described (11). One unit of calpain activity is defined as the amount of enzyme that causes the release of 1μmol of free ammino groups per hour, To ascertain whether the formation of the PKI55-PKC complex affects the PKC molecule's properties, we studied its digestibility by the calcium-dependent proteinase calpain (28-29). As shown in Figure 7A, calpain degraded PKC and in 1 hour of incubation converted the native enzyme's form into that of PKM, the free catalytic fragment that is active in the absence of any cofactors (filled circles). In the course of the digestion, no apparent loss of PKC activity was detected (filled triangles). With the addition of PKI55 (Figure 7B), conversion into PKM is accelerated but PKC is also degraded to an inactive fraction, as can be deduced from the reduced amounts of detectable enzyme during incubation. Addition of PKC cofactors together with PKI55 further accelerates both the conversion and the inactivation of the kinase (Figure 7C). In these conditions, PKI55 proved fully resistant to calpain action and no loss of inhibiting capacity was detected. These data indicate not only that PKI55 inhibits activated forms of PKC, but also that it induces a conformational state in PKC, which is much more sensitive than the native form to digestion by calpain to an inactive fraction, as can be deduced from the reduced amounts of detectable enzyme during incubation

Library Screening

Three cDNA human libraries: a fetal spleen library, an infant brain library and a HL-60 library were screened by hybridisation with the coding portion of KI-55 amplified by PCR using

5'-ATGCTGTATAAACTGCATGA-3' and poly-dT primers. The positive clones were sequenced by an ALF automated sequencer (Pharmacia).

A CEPH YAC genome library obtained from theYAC Screening Centre of DIBIT (Milan) was tested in pooled samples suitable for analysis using PCR. PCR was performed using the following primers: 5'-AGGCTGTAGAGAGGATTGAG-3' and 5'-ATGGTCCTGTGGTGTTGG-3'. Pulse-field gel electrophoresis (PFGE) of genomic DNA obtained from positive YACs was performed. Yeast DNA in an agarose plug was prepared, loaded onto 1.25% Seakem GTG and was run at 200V at 148C in 0.5x TAE. Yeast DNA was transferred to a nylon filter and hybridized with the 168bp coding portion of PKI55 by random priming.

Electroporation

$1xI0^7$ cells were centrifuged for 5' at 500g and washed with PBS 1x. Pellet is then resuspended in pellet in 0.8 ml of Iscove's medium and transferred to sterile cuvet (Gene Pulser - Biorad). 15 pg of the DNA plasmide. After 15 min incubation on ice cells were exposed to a 0.25 kV electric field - 960 JXFD (Gene-Pulser, BioRad). After further 15 min in ice cells were resuspended in culture medium.

Example 1. Identification of the novel gene KI55.

A degenerated oligonucleotide was designed on a 5-amminoacid sequence of IN (21). A 168 bp amplification product, was obtained in the course of RT-PCR experiments. No sequence homology between the 168 bp PCR product and catalogued genes was found in a Genbank search.

The 168 bp PCR product used as a probe, hybridized in a human fetal spleen cDNA library a positive clone of 833 bp that included the 168 bp sequence. A search in dbEST revealed a complete sequence identity of the 833 bp sequence to several ESTs, all of which were included in the 1530 bp long I.M.A.G.E. clone ID 38900 (accession numbers R51337 and R51448) (22). The 1530bp cDNA contains 6 ORFs, of different length, in the three positive alternative reading frames (ORF Finder program from http://www.ncbi.nih.nlm.com).

A major ORF in the first reading frame (SEQ ID NO 1) shows an AUG in position 1330 that was the "first" in an adequate genetic context, carrying a purine (A) at -3 and a pyrimidine (C) in +4; the 253 bp of leader length should grant a satisfactory translation and prevent the interference of the cap on the initiator codon; it is moderately structured as shown by 51% G+C content and no hairpin structures have been detected; the downstream secondary structure accounts for 52% of G+C content and the coding sequence includes, in the appropriate reading frame, the 168 bp amplification product previously detected (23-26). Transcription-translation experiments demonstrated that the 1530 bp cDNA of IMAGE clone produced a translation product of 6.5 kDa, corresponding to 55aa coded by the ORF with the first AUG in position 1330 (Fig. 1, SEQ ID NO 2). The translation of the conceptual reading frame of this ORF allowed the preparation of a synthetic protein, that was found to act as a PKC inhibitor.

The coding sequence and the coded peptide werenamed Protein Kinase Inhibitor 55 (PKI55).

Localisation of KI-55

FISH analysis performed as described in Materials and Methods revealed that the hybridization of a metaphasic preparation of human T-lymphocytes with YAC clone 797A11 yielded a single signal localized in chromosome 2q35

Bioinformatic study of KI55

A Bioinformatic analysis of chromosome 2q35 was performed at the address http://genome.ucsc.edu visiting EMBL at "Ensemble Contig view "Chr. 2q35" sequence is shown between 223-235Mbp.

The 2q35 BAC Clone RPCI11-1064L18 (AC008123) (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide), 168601bp long, is localized on chr. 2q35 between 225825265 and 225993866 bp.

We used for analysis the multiprogram NIX (from: http://www.hgmp.mrc.ac.uk). The main results were: a 533 bp LTR32/ERVL region identified between 88270 and 88803 bp of the BAC Clone RPC I11-1064L18 (Fig. 2). This region includes completely PKI55: the coding sequence ranges between 88535 and 88701 bp, it has a starting and a terminal codons (SEQ ID NO 1). A promoter sequence has been identified at 88431 (-102bp from the first AUG of the coding sequence) and a

TATA box at 88406 (-25bp from the promoter).

Several consensus sequences for common transcription factors, probably representing an enhancer element, have been identified from nucleotide 88130 to nucleotide 88534 (Fig. 2). Two AP1 transcription factor consensus sequences have been identified: the first at 87379bp (-1052bp from the promoter) in the Responding Elements Region (RE) and, a second one, in a less canonical position at 88456 (+25bp) from the promoter. Two analyses were carried out.

In the first we made a similarity search of the available sequences databases with an amminoacid query sequence of PKI55, using the tblastn program 2.2.1 NCBI (27). The database tested included 958.081 protein sequences and 4.118.683.734 total letters. In this way, the alignment of the ammino acids in the sequence, the score of matches in the two sequences, the identities, positives, gaps count and the E parameter were obtained. The latter quantifies the number of random hits that are expected, when searching a database of a given size. Two evaluation criteria were then applied: one, more restrictive, rejects the random hits hypothesis if the tabulated value is less than the inverse of the number of ammino acids present in the database ($4x10^{-9}$); the second, less restrictive criteria, rejects the random hits hypothesis when the tabulated value is less than the inverse of the number of protein sequences in the database ($1x10^{-6}$).

In the second analysis a pairwise BLAST nucleotide sequence was performed with a nucleotide query sequence of the 2661bp cDNA from CloneDKFZp434H1419 (AL137534) that is included in 2q35BACRPCI11-1064L18 Clone from bp 86123 to bp 88735. It is therefore comprehensive of the KI55 coding sequence and regulating region.

Similarities of the PKI55 amminoacid sequence tested with tblastn program were found in 50 clones, included in 18 chromosomes and in a Macaca Fascicularis brain cDNA.

The latter's score was 102, with 83% maximum degree of identities, 88% degree of positives and E 10^{-21}. The human chromosomes 10, 15 and 20 show highly significant similarities of the amminoacidic sequence (Fig. 9) Macaca Fascicularis and the three Chromosomes above (SEQ ID NO 3, 6, 8, 10), show also significant similarities of the nucleotide sequence in the 5' regulating region

Pairwise BLAST Nucleotide Sequences

Fig. 9b describes the query sequence cDNA from Clone DKFZp434H1419 (AL137534): genomic organization of PKI55, Macaca Fascicularis and three paralogs, located on Human chromosomes 10,15,20 (SEQ ID NO 1, 3, 6, 8, 10).

Similarities to the PKI55 amino acid sequence, tested with tbastn program, were found in 50 clones contained in 18 chromosomes and in a Macaca Fascicularis brain cDNA Clone QnpA-21012 .The latter's score was 102, with 83% maximum degree of identities, 88% degree of positives and E 10^{-21} (Fig. 9a).

Human chromosome 10 Clone RP11-36N22, human chromosome 15 Clone RP11-57P19 and human chromosome 20 Clone RP4-697P8 contain sequences that bear highly significant similarities to the amino acid sequence of PKI55 (Fig. 9a) of Macaca Fascicularis brain cDNA. The three chromosomes mentioned above also showed significant similarities to the nucleotide sequence in the 5' regulating region (Fig. 9b). Fourteen other human chromosomes (1, 4, 5, 6, 7, 8, 11, 12, 13,14, 16, 17, 22 and X) showed extensive rearrangements of the coding sequence and of the 5' regulatory region. The remaining six human chromosomes (Y, 3, 9, 18, 19 and 21) resulted completely negative according to both the Blast tests.

Not horizontal transmission through infection between Macaca Fascicularis and humans but, rather, the direct derivation of the two species from a common ancestor that existed before the divergence between Old World Monkeys and Humans occurred some 25 Myr ago, appears to be the most likely explanation of the presence of the KI55 gene in both lineages. This assumption is compatible with the fixation of HERVL sequences in the genome of primates, before the apparent death of ERVL and MaLRs elements occurred some 40 Myr ago (36, 44). The KI55 gene in 2q35 (SEQ ID NO 1) appears to be the ortholog derived by speciation in the human genome. The gene became subsequently diffused in the human genome by transposition through the LTR32-ERVL sequences. The very great similarities in amino acid sequence and noteworthy similarities in nucleotide sequence of 5' regulating region of PKI55 (Fig. 9) of Macaca Fascicularis brain cDNA and the three chromosomes mentioned above indicate a selective advantage of the gene structure.

NMR study

The 1H-ROESY NMR spectra do not show any evidence of meaningful connectivities through space between protons that can be associated with tertiary structures. The results of the CD analysis exclude the presence of helicoidal arrangements in the polypeptide chains, thus supporting the hypothesis that the structure of PKI55 is completely random. The relatively low affinity of PKI55 for its PKC targets (around 10-5 M) may be due to the disordered structure of the protein inhibitor. The latter permits multiple molecular conformational forms.

Table 1. Effect of PKC cofactors on PKI55 inhibitory efficiency

Human recombinant PKC α (A) was assayed as described in Methods in the presence of the indicated cofactors with or without 6μM PKI55 (SEQ ID NO 2). Human recombinant PKC δ (B) was assayed with or without 6μM PKI55 (SEQ ID NO 2). PKC activity was expressed as a percentage of the activity measured as units/ml. in the presence of all co-factors, without PKI55.

TABLE 1. Effect of PKC cofactors on the PK155 inhibitory efficiency

PKC isoform	Cofactor added				PKC ACTIVITY % (units/sample)		I %
	CA^{2+}	PS	DAG	ATP	-PKI55	+ PKI55	
α	+	+	+	+	2,56	0,81	68
α	+	+	-	+	2,4	0,8	67
α	+	+	+	½	1,9	0,67	65
α	-	+	+	+	0,11	0,1	10
α	-	-	-	+	0,01	0,01	---

δ	+	+	+	+	3,2	1,17	64
δ	+	+	-	+	2,2	0,78	65
δ	+	+	+	½	2	0,76	62
δ	-	+	+	+	3,2	1,17	64

Fourteen human chromosomes 1, 4, 5, 6, 7, 8, 11, 12, 13, 14, 16, 17, 22 and X show extensive rearrangements of the coding sequence and of the 5' regulating region. The remaining six chromosomes were completely negative at both the Blast tests.

PKC-Inhibiting Properties of PKI55

Recombinant PKI55 (rPKI55) (SEQ ID NO 2), prepared in E.Coli was tested on human recombinant PKCα and PKCδ isozymes that were inhibited; rPKI55 was ineffective on cAMP-dependent PKA.

The Eadie-Hofstee plot of PKI55 inhibition on PKCα. is a straight line, which indicates the absence of cooperative effects and the existence of one or more independent PKI55 binding sites. The K_d is calculated to be approximated in 14μM (Figure 4A).

PKI55 inhibitory efficiency on PKCδ isozyme is shown in Figure 4B; the plot of PKCδ inhibition by PKI55 is a straight line with a K_d of approximately 13μM.

We tested PKI55 activity after removing each cofactor, in turn. As shown in Table 1 PKC activity was inhibited to the same extent, independently of which cofactor was removed from the incubation mixture, with the exception of Ca^{2+}. It is well known that Ca^{2+} induces in cPKCs a molecular transition at the level of the regulatory domain: the transition, presumably, leads to the onset of the active state, which is then recognized by PKI55. This conclusion is supported also by the absence of any inhibitory effect with or without Ca^{2+}, on PKM, the PKC form that has been deprived of the regulatory domain. As expected, the PKI55 effect on δ isozyme is essentially insensitive to Ca^{2+} treatment. Taken together, these data strongly suggest that cPKCα inhibition by PKI55 is triggered by a conformational change of the kinase, unnecessary in the case of nPKCδ and that both are independent from the cofactors.

PKC-PKI55 Molecular Complex

When non-activated PKCα isozymes were electrophoresed in presence of sPKI55 (SEQ ID NO 2) and Western blotted with anti-PKI55 and with anti-PKC antibodies, two separate bands were visible, at the specific molecular weight of the two proteins. Viceversa, a single band of 87 kDa is detected when added PKCα isozymes were previously activated (Fig. 5 lane a' and a").

Cell lysates of PMA and PHA treated PBMC were immunoprecipitated with anti-PKI55 antibody and Western blotted with anti-PKCα antibody (Fig. 6A), a single band of 87kDa was detected.

The reciprocal experiment using anti-PKCα as the precipitating antibody, followed by Western blotting with anti-PKI55, also showed a single band of 87 kDa (Fig 6B). These experiments show that PKI55 interacts only with activated PKCα and coimmunoprecipitation results point to the existence of macromolecular complex between PKI55 and activated PKCα isozymes.

The 87 kDa molecular mass of the complex detected by both the anti-PKI55 and anti-PKCα antibodies in the coimmunoprecipitation experiments, corresponds to the sum of the PKC molecular mass (80 kDa) plus PKI55 molecular mass (6.5 kDa), suggesting a 1:1 stoichiometry.

In vivo experiments

The in vivo action of PKI55 has been evaluated by three sets of experiments

1) The recombinant PKI55 (SEQ ID NO 2) purified from cell lisate of bacterial cultures was assayed in vivo on PKC semipurified from citosol. The IC50 of this test was 100 nM.

The recombinant PKI55 (1 μM) was added to culture medium of PHA stimulated human Lymphocytes. The activity of the accumulated PKC was severely reduced.

These experimental results demonstrate that: PKI55 has an inhibitory activity of on PKC in human cells; PKI55 is active against alfa, beta I and beta II and delta isozymes.

Moreover it is also shown that PKI55 is able to cross the cell membrane.

2) The confocal work Fig 13 has clearly demonstrated that Fluoroscinated sPKI55 added to the culture medium can cross cell membrane confirming the observations described in 1)

3) By means of a polyclonal Antibody against sPKI55 (Fig14) it has been possible to demonstrate that PKI55 is produced by activated PBMC (PHA,OKT3) and is detectable in cell lysates. Furthermore PKI55 is detected also in the conditioned medium confirming that the protein can cross the cell membrane in both directions: in and out, of the cell.

Regression analysis of antibody against known doses of sPKI55
leads to quantitative estimates of PKI55 in the Test samples.

PKI55-FITC was used to demonstrate that PKI55 could penetrate the cytosolic membrane. Confocal immunofluorescence analysis was performed in permeabilized and nonpermeabilized lymphocytes, both in resting state and after activation with PMA and Ca^{2+}-ionophore A23187. As shown in Figure 13, when the cells were permeabilized both PMA and Ca^{2+}-ionophore A23187 treated cells showed a significant immunofluorescence. When the cells were not permeabilized, PKI55-FITC penetrated the cell membrane only after Ca^{2+}-ionophore A23187 activation.

Feedback PKC-PKI55 system.

The synthesis and accumulation of PKI55 were analyzed after cellular stimulation (42). In the present study, we used unstimulated PBMC, PMA, which selectively triggers the PKC pathway, and OKT3 or PHA, which stimulate both the PKC and the calcium cascade pathways

In lysates of unstimulated PBMC, a PKI55 6.5 kDa (SEQ ID NO 2) band is occasionally detectable (1/20), while a tiny 87 kDa band is always visible, indicating previous PKC activation events (Fig. 8a6a lane1). In PHA-, OKT3- and PMA-stimulated PBMC lysates, the 87 kDa molecular complex is present in largely increased quantities and a 6.5 kDa band usually becomes visible (Fig. 8a6a lanes 2, 3, 8). Activated PBMC cultures were treated with cycloexamide, a known inhibitor of protein synthesis and only the 87 kDa complex was present in the cell lysates (Fig. 8a6a lanes 6, 7, 10) of cycloexamide treated cells.

This finding suggests that the activating treatments induce de novo synthesis of PKI55 that is inhibited by cycloexamide. When PHA-, OKT3- and PMA-stimulated PBMC cultures were treated with the specific PKC inhibitor H7, the PKI55 6.5 kDa band was not visible, indicating that the peptide synthesis is inhibited (Fig. 8a6a

lanes 4, 5, 9). These findings provide evidence that de novo PKI55 synthesis is PKC dependent.

PKC activated by cofactors is effective for a short time and rapidly returns to an inactive state. When PMA activation was prolonged to 72 hrs only the 87 kDa complex was visible throughout the 48 hrs of culture: neither native PKI55 (6.5 kDa) nor 80 kDa PKC were detected (Fig. 8b6b). After 72 hrs of culture the 87 kDa complex is reduced to 5% (11.5 to 0.5 DU); 80 kDa PKC and native PKI55 band (6.5 kDa) also become visible (Fig.8b 6b)

The 45 kDa band corresponding to the PKC degradation product PKM is visible after 30 min to 72 hrs of incubation time.

Activity of a peptide derived from PKI55 in an experimental model of ischemic damage

Two different synthetic peptides were produced based on the sequence of the PKI55 (SEQ ID NO 2) protein (named G16 and F20).

G16: MLYKLHDVCRQLWFSC

F20: PACHHRAMRICCPAQHHRTI

The aim of the prosecution of the study of Peptides reduced in number of Aminoacids (herein Reduced Peptides: RP) is to better define the PKC consensus sequence and the role of the flanking regions to identify the nature of the residue involved in the formation of the covalent bond PKI-PKC. We will shorten the peptides from both the Nh2 and COOH side of the molecule. IC50 comparisons among the RPs will be examined in a dose dependent test against Alfa and Delta isozymes.

RPs will be selected with IC50 as small as possible and in any case no greater than13-microM for alfa and 14microM for delta PKC isozymes with any number of aa, not lower than 3 and not higher than 55. Optimal range expected 5-8 aa.

NMR analysis and the tertiary structure will be examined in the selected RPs.

Several advantages of the reduction of the active peptide size are expected: increased solubility and penetrability in the cell; increased efficacy and decreased doses with a potential bigger selectivity of PKC isozymes; preparation of D-peptides more resistant to eso–peptidases; and most important, possibility to insert or to substitute for functional groups more reactive.

Hopefully these manipulations will lead to the preparation of a "drug" potentially very usefull in the therapy of important human diseases, where the partecipation of "Kinases' deregulation" is suspected.

Each peptide was tested in the cytosolic semipurified PKC from Human Lymphocytes in dose dependance raging from 0.1 mM to 100mM.

The G16 peptide, producing a 90% inhibitory effect with an IC50 of 50 uM in the assay of cytosolic PKC, was choosen to study the effect of derived peptides on the acetilcoline release in slices of rat brain under in vitro iskaemic damage.

In normal condition of perfusion the peptide in presence of normal levels of O2 and glucose does not change the aceticoline release under electrical stimulation. After ischemic damage produced by substitution of O_2 with N_2 and by glucose deprivation of the perfusion medium the release of acetilcoline is decreased and the decrease persist during the observation period.

In presence of the G16 peptide the decreased release of acetilcoline shown a trend to normalization in about 2 hours. The decreased acetilcoline observed in untreated control is similar to that observed in sample treated with the F20 inactive peptide. Samples treated with this inactive peptide do not show a return to normal values.

These experimental results shows that: the active G16 peptide has a recovery effect on the iskaemic damage in rat brain slices at least in this experimental model.

Also the petide seems able to cross the cell membrane in these experimental conditions.

<u>Effect of pKI55 transfection in Jurkat cells</u>

To have a preliminary evaluation of the PKI55 action on the cell growth in tumor cells, experiments were performed involving the transfection of the KI55 (SEQ ID NO 1) gene in Jurkat cell (ATCC) a lymphoblastoid cell line derived from a T cell leukemia.

We measured the cell growth (by Trypan blue exclusion counts, as shown in Fig.11) in cells transfected with: pRc/RSV plasmid inserted with the gene in the right direction (pRc/RSV- senso) with the plasmid alone (pRc/RSV, Invitrogen) and with the plasmid containing the gene in the antisense (pRc/RSV-antisense).

FACS analysis was used to evaluate the cell cycle position of the cells.

All the cell cultures showed an exponentially cell growth however the cells transfected with the pRc/RSV-senso plasmide reached the plateau phase at a cell concentration that is almost 50% of the plateau concentration of control cells. A reduced cell concentration at plateau was observed also in cell transfected with pRc/RSV .

Facs analysis showed that only in the Jurkat cells transfect with pRc/RSV-senso there is a significant accumulation in the G2/M phase of the cell cycle.

The Western Blotting and PKC assay showed that the specific activity of the PKC isozyme beta I is substantially decreased in comparison to the activity observed in the other cells.

The isozyme beta II has a reduced activity in cells transfected with pRc/RSV-senso and pRc-senso in comparison with controls and cells transfected with pRc/RSV-antisenso

The isozyme alpha is reducded in cells trasfected with pRc/RSV-senso and in cells trasfected with pRc/RSV.

These experimental results show that:

PKI55, obtained by transfection of the coding gene in Jurkat cells, is able to cause a G2/M block that is consistent with a reduced activity of the PKC, especially of the beta II isozyme.

The pRC/RSV plasmide was used to insert the DNA coding for the protein PKI55 in sense and antisense direction: restriction analysis and sequencing were used to control the plasmids. Jurkat cells were transfected by electroporation. A reporter plasmid pCV-Rgal was used to evaluate the percentage of transfected cells with a α-galactosidase test performed at 48 hrs.

The structure of the plasmid is illustrated in Fig 10.

<u>Expression of PKI55 protein in cells from a group of patients suffering by non asmatic allergic rhinitys</u>

In order to have preliminary indications of the importance of the PKI55 protein in some disease we decided to test the expression of this protein (SEQ ID NO 2) in Lympocyte from rhynitis patients before and after PHA stimulation. Western blotting and PKC assay was applied to detect the expression of the PKI55.

Contrary to the observation in lymphocytes from healthy subjects, PKI55 was undetectable in cell lysates before and after PHA stimulation. The protein was also undetectable in concentrated colture surnatant (Tab. 2).

These experimental results suggest that a prolonged PKC activation without the regulation of the feed-back mechanism based on PKI55 may have an important pathogenetic role in the allergic rhinitys.

Tab. 2 Western blotting assay of PKI55 expression unstimulated and PHA stimulated lymphocites from non asmatic allergic rinitis patients.

Tab. 2

	Anti PKI-55 detection		
Rinitis patients	PBMC Lysates Unstimulated	Lysates Pha-activated	Surnatant concentrated
1	neg.	Neg.	neg.
2	neg.	Neg.	neg.
3	neg.	Neg.	neg.
4	neg.	Neg.	neg.
5	neg.	Neg.	neg.
6	neg.	Neg.	neg.
7	neg.	Neg.	neg.
8	neg.	Neg.	neg.
9	neg.	Neg.	neg.
10	neg.	Neg.	neg.
11	neg.	Neg.	neg.
12	neg.	Neg.	neg.

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CLAIMS

1. An isolated polypeptide characterized in that it binds to activated Protein Kinase C and in that it shows an aminoacid sequence identity of at least 55% to sequence IDN 2.

2. Polypeptides according to claim 1 wherein said binding modulates PKC activity

3. Polypeptides according to claim 2 wherein said protein kinase C is a alpha, beta I, beta II, gamma, delta, epsilon, lambda, theta, mu, eta and zeta PKC isoform.

4. Polypeptides according to claim 3 wherein said protein kinase C is selected from the isoforms alpha or delta

5. Polypeptides according to claim 4 selected from the group consisting of: seq IDN 2, 4, 5, 7, 9, 11, 12 and 13.

6. Polypeptide according to claim 1 having an apparent molecular weight of 6.5 Kd

7. A functional subsequence comprising at least 4 aminoacids of the polypeptides according to claims 1-5.

8. The functional subsequence of claim 7 optionally carrying one or more amino acid modifications.

9. The functional subsequence of claim 8, wherein said modification comprises an aminoacid substitution, deletion or insertion.

10. The functional subsequence of claim 9, wherein said substitution comprises a conservative amino acid substitution.

11. The fuctional subsequence of claim 10 characterized in that it comprises at least 4 aminoacid of the N-terminal region of sequences selected from the group consisting of: SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO6, SEQ ID NO 7, SEQ ID NO 9, SEQ ID NO 11, SEQ IDN 13.

12. The fuctional subsequence of claim 11 corresponding to the following aminoacid sequence derived from seq IDN2:

1-4: MLYK

5-10: LHDVCR

1-10: MLYKLHDVCR

1-16: MLYKLHDVCRQLWFSC (seq IDN13)

1-26: MLYKLHDVCRQLWFSCPACHHRAMRI (seq IDN12)

13. The functional subsequence of claim 11, said subsequence having from about

10 to about 50 amino acids in lenght.

14. Isolated polypeptide according to claim 1-6 or functional subsequences according to claim 7-13 comprising at least one aminoacid substitution wherein said substitution is of an aminoacid in configuration L- with an aminoacid in configuration D.

15. The polypeptide of claims 1-6 or a functional subsequence according to claim 7-13 further comprising a heterologous functional domain.

16. The polypeptide of claim 15, wherein said heterologous domain comprises an amino acid sequence.

17. The polypeptide of claim 16, wherein said heterologous domain comprises a tag.

18. The polypeptide of claim 15, wherein said heterologous domain comprises a detectable label.

19. An antibody or fragment thereof characterized in that it specifically binds to the polypeptide of claims 1-6 or to an immunogenic fragment thereof.

20. The antibody of claim 19, wherein the polypeptide has the aminoacid sequence set forth in SEQ ID NO: 2, 4, 5, 7, 9, 11, 12, 13.

21. A method for producing an antibody that specifically binds to the polypeptide of claims 1-7 or to the functional polypeptide according to claims 7-13, comprising administering said polypeptides, or a polynucleotide encoding the same, to an animal in an amount sufficient to produce an antibody that specifically binds to the polypeptide of claims 1-6, 14-18 and 7-13.

22. An isolated or recombinant polynucleotide encoding a polypeptide of claims 1-6 or a subsequence thereof.

23. The isolated polynucleotide according to claim 22, wherein the polypeptide has the sequence set forth in SEQ ID NO: 2, 4, 5, 7, 9, 11, 12, 13.

24. An isolated or recombinant polynucleotide according to claim 22 encoding a polypeptide with at least 50% identity to the polypeptide of SEQ ID NO: 2, or a subsequence thereof.

25. An isolated or recombinant polynucleotide according to claim 24 selected from the group consisting of:

a) SEQ ID NO: 1, 3, 6, 8, 10;

b) SEQ ID NO: 1, 3, 6, 8, 10 wherein one or more T's are U;

c) nucleic acid sequences complementary to a) or b); and

d) subsequences of either a), b) or c) that are at least 15 base pairs long.

26. An isolated or recombinant polynucleotide that hybridizes under moderately stringent conditions to the sequence set forth in SEQ ID NO: 1.

27. An isolated or recombinant polynucleotide that hybridizes under highly stringent conditions to the sequences set forth in SEQ ID NO: 1, 3, 6, 8, 10.

28. A promoter region comprising all or part of the 5' region preceding the coding region set forth in SEQ ID NO 1, 3, 6, 8, 10.

29. An isolated or recombinant polynucleotide comprising a nucleic acid having a degree of homology ranging from at least 50% to 100%, as determined using a BLAST algorithm, to a sequence corresponding to SEQ ID NO: 1, 3, 6, 8, 10.

30. Recombinant isolated polypeptides characterized in that they are obtained by expression of the isolated polynucleotides according to claims 22-27.

31. An expression cassette comprising a nucleic acid sequence operably linked to the polynucleotide according to any of claims 22-27.

32. A vector comprising the expression cassette of claim 31 or the isolated polynucleotides according to claims 22-27.

33. A host cell containing the vector according to claim 32.

34. A method for modulating PKC activity in a cell comprising contacting a cell with an amount of polypeptide of claim 1-6 or 14-18, a functional fragment according to claims 7-13, a nucleic acid according to claims 22-27, or an antisense or complementary to said polynucleotides claim.

35. A method according to claim 34 wherein said PKC is a delta or alpha PKC isophorm

36. A method for identifying a compound that modulate PKC activity, comprising

a) incubating a test compound with polypeptides according to claims 1-6 or 14-18 under conditions allowing binding to PKC; and

b) detecting whether the test compound inhibit said binding by direct or indirect assays.

37. A method according to claim 37 wherein said PKC is PKC alpha, beta I, beta II, gamma, delta, epsilon, lambda, theta, mu, eta and zeta.

38. A method for identifying a compound that modulates the activity or the expression of any of the polypeptides according to claims 1-6 or 14-18, comprising:

a) incubating a test compound with any of the polypeptides under conditions allowing binding; and

b) determining said polypeptides activity or expression of a nucleic acid encoding polypeptide 1-6 or fragments thereof, in the presence of the test compound, wherein an increase or decrease in said activity or expression identifies the test compound as a compound that modulates polypeptides according to claims 1-6 activity or expression.

39. Polypeptides according to claims 1-6 or functional subsequences according to claims 7-13 for use as a medicament.

40. Polypeptides according to claims 1-6 or functional subsequences according to claims 7-13 for use for the prevention or treatment of diseases characterized by PKC disregulation.

41. Polypeptides according to claim 40 wherein such diseases are cell proliferation disorders.

42. Polypeptides according to claim 40 wherein said diseases are chosen among: autoimmune, allergic, oncologic and neurologic disorders.

43. Polypeptides according to claim 42 wherein said neurologic disorder is an iskaemic damage.

44. Polypeptides according to claims 1-6 or 14-18 or functional subsequences according to claims 7-13 and/or antibodies according to claims 19-20 for diagnostic use.

45. Composition comprising at least one of the polypeptides according to claims 1-6 or functional subsequences according to claims 7-13 for use as a medicament.

46. Composition comprising a therapeutically effective amount of at least one of the polypeptides according to claims 1-6 or functional subsequences according to claims 7-13 for use for the prevention or treatment of diseases characterized by PKC disregulation.

47. Composition according to claim 46 wherein such diseases are cell proliferation

disorders.

48. Composition according to claim 46 wherein said diseases are chosen among: autoimmune, allergic, oncologic and neurologic disorders.

49. Composition according to claim 48 wherein said neurologic disorder is an iskaemic damage.

50. Composition according to claims 47-51 further comprising at least an additional component selected in the group of: adjuvants, eluents, eccipients, carriers, diluents, fillers, salts, buffers, stabilizers, solubilizers.

51. Composition according to claims 45-50 in the form of tablets, capsules, powders, solutions, elixir, pills, dragees, liquids, gels, syrups, slurries, suspensions

52. Diagnostic agent comprising at least one of the polypeptides according to claims 1-6 or 14-18 or the functional subsequences according to claims 7-13.

53. Use of at least one of the polypeptides according to claims 1-6 or the functional subsequence according to claim 8-14 to prepare a medicament for the treatment or the prevention of diseases characterized by PKC disregulation.

54.Use according to claim 53 wherein such diseases are cell proliferation disorders.

55. Use according to claim 53 wherein said diseases are chosen among: autoimmune, allergic, oncologic and neurologic disorders.

56. Use according to claim 55 wherein said neurologic disorder is an iskaemic damage.

57. Polynucleotides according to claims 22-27 for diagnostic use.

58. Polynucleotides according to claims 22-27 for use as a medicament.

59. Composition comprising at least one of the polynucleotides according to claims 22-27 for use as a medicament.

60. Composition comprising a therapeutically effective amount of at least one of the polynucleotides according to claims 22-27 for use for the prevention or treatment of diseases characterized by PKC disregulation.

61. Composition according to claim 60 wherein such diseases are cell proliferation disorders.

62. Composition according to claim 60 wherein said diseases are chosen among: autoimmune, allergic, oncologic and neurologic disorders.

63. Composition according to claim 62 wherein said neurologic disorder is an iskaemic damage.

64. Composition according to claims 59-63 further comprising at least an additional component selected in the group of: adjuvants, eluents, eccipients, carriers, diluents, fillers, salts, buffers, stabilizers, solubilizers.

65. Composition according to claims 59-63 in the form of tablets, capsules, powders, solutions, elixir, pills, dragees, liquids, gels, syrups, slurries, suspensions

66. Diagnostic agent comprising at least one of the polynucleotides according to claims 22-27.

67. Use of at least one of the polynucleotides according to claims 22-27 to prepare a medicament for the treatment or the prevention of diseases characterized by PKC disregulation

68. Use of the polynucleotides according to claim 67 wherein such diseases are cell proliferation disorders

69. Use of the polynucleotides according to claim 67 wherein said diseases are chosen among: autoimmune, allergic, oncologic and neurologic disorders.

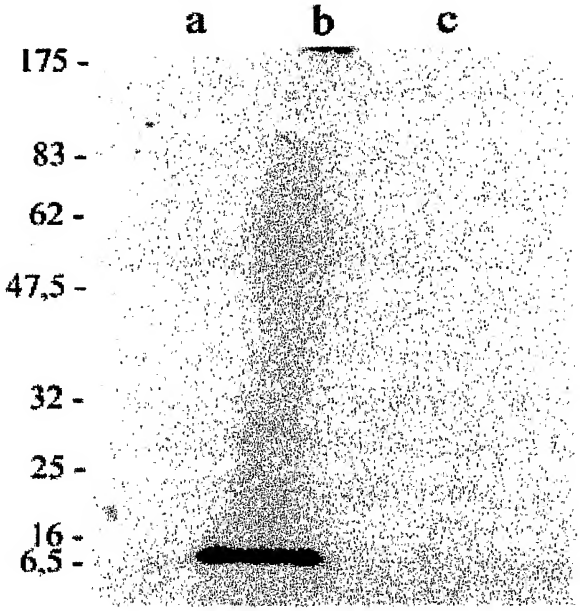
70. Use of the polynucleotides according to claim 69 wherein said neurologic disorder is an iskaemic damage

71. A method for the detection of allelic variations, mutants or single nucleotide polymorphisms of sequences ID NO 1, 3, 6, 8, 10 comprising:

a) incubating a test DNA with any of the polynucleotides of claims 22-27.

b) determining the degree or the lack of similarity between the test DNA and the polynucleotides

Figure 1.



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AP1
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87601 gctttcctag ggcatcttct tataagacac cagtcgtata aggagcccac cccactcatc

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87961 cacctgaaat atgggactca atcattttag tccccttctt tctataagtg tgtgtgtgtg

88021 gatatgtgtg ctagatgttc ttgctgtgtt aggaggtgat aaacatttgt ccatgttata

88081 taggtggaaa gggtcagact actaaattgt gaagacatca tctgtctgca tttattgaga

88141 atgtgaatatgaaacaagct gcaagtattc tataaatgtt cactgttattagatattgta

88201 tgtcttttgtgtcctttttatt catgaattct tgca cattat gaagaaagag tccatgtggt

88261 cagtgtctta *cccggtgtag ggtaaatgca cctgatagca ataacttaag cacacctta*

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TATA Box Prom.
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AP1
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88501 gtgtcgctca ttagggaaag gggttaagtg aaa[b] atgctgtataaactgcatgatgtttgc
M L Y K L H D V C
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R Q L W F S C P A C H H R A M R I C C P
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A Q H H R T I S V C K T I L S S P P P L
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D S L P C M u
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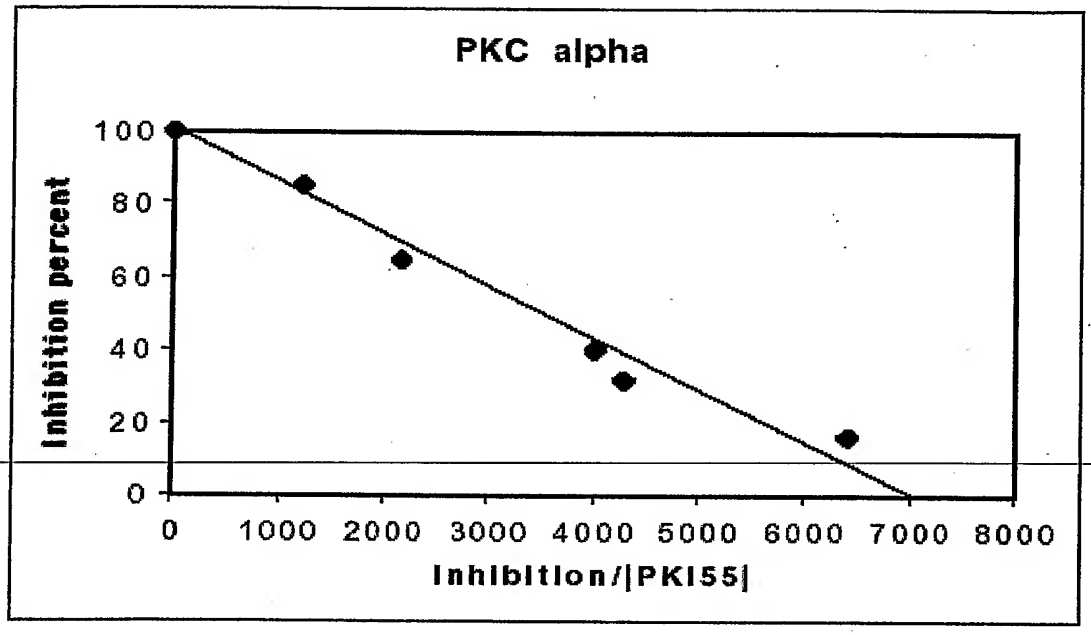
88801 aca

Figure 2

A)


PKC alpha
Inhibition percent
100
80
60
40
20
0
0 1000 2000 3000 4000 5000 6000 7000 8000
Inhibition/|PKI55|

B)

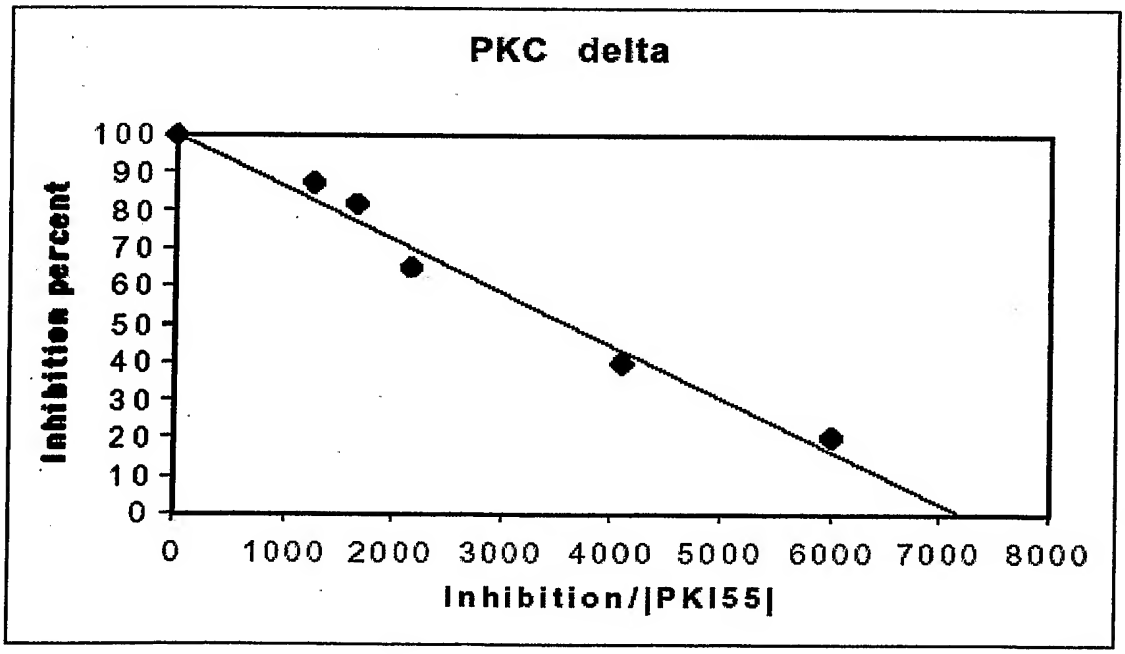
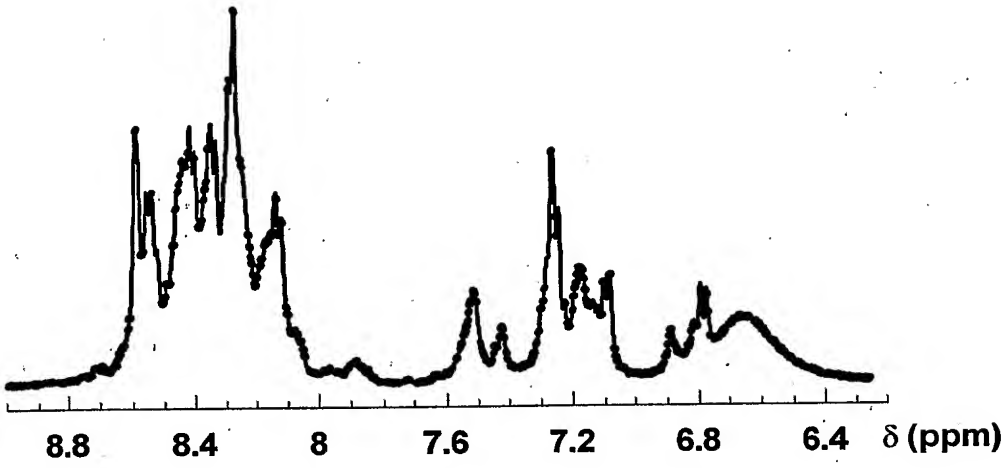

PKC delta
Inhibition percent
100
90
80
70
60
50
40
30
20
10
0
0 1000 2000 3000 4000 5000 6000 7000 8000
Inhibition/|PKI55|

Figure 3

Figure 4



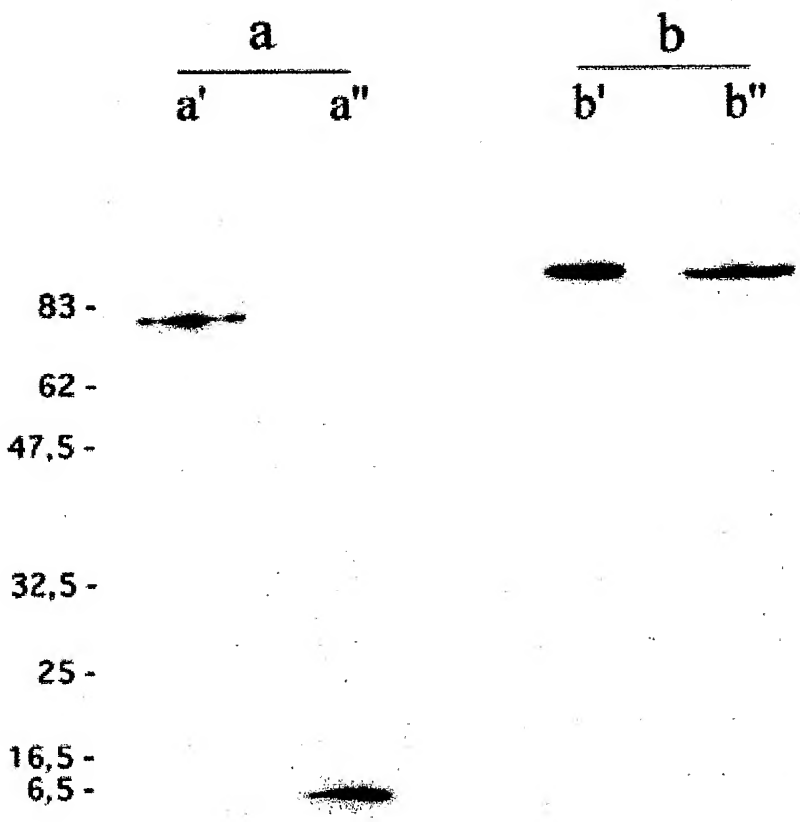
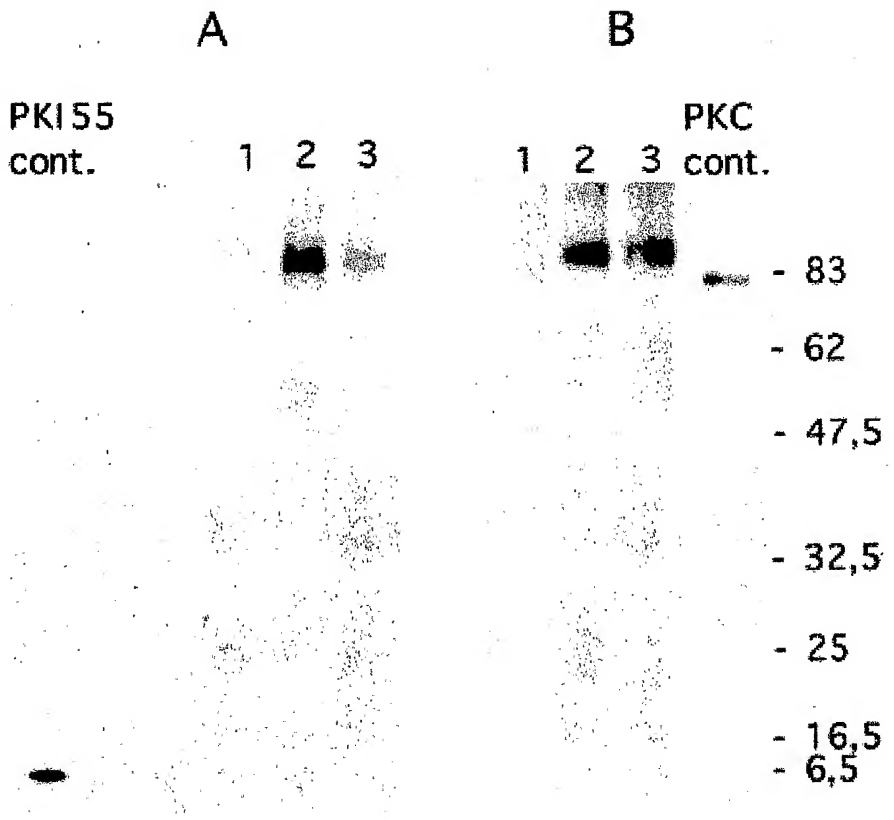


Figure 5



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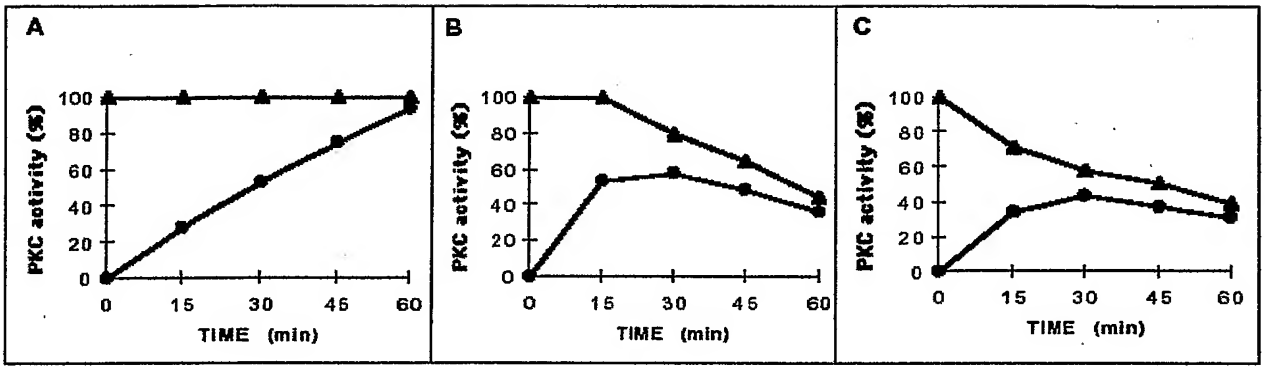
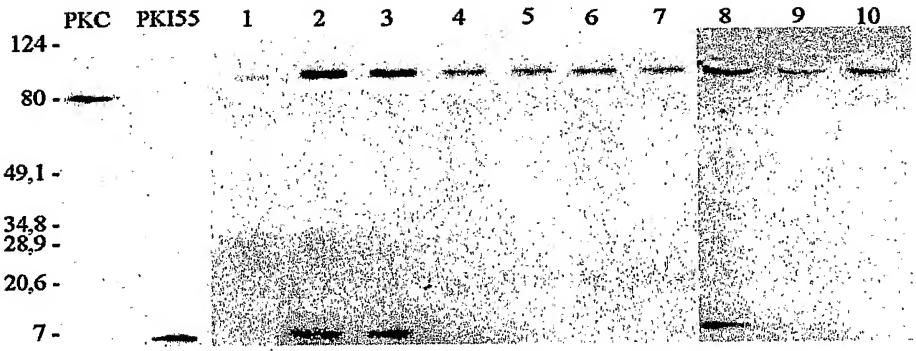


Figure 7

a)


PKC
PKI55
1
2
3
4
5
6
7
8
9
10
124 -
80 -
49,1 -
34,8 -
28,9 -
20,6 -
7 -

b)

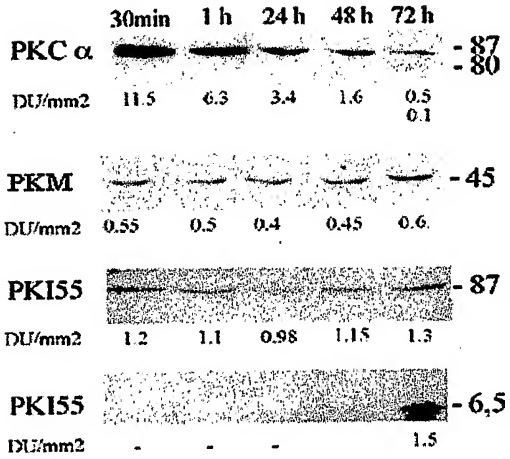

30min
1 h
24 h
48 h
72 h
PKC α
- 87
- 80
DU/mm2
11.5
6.3
3.4
1.6
0.5
0.1
PKM
- 45
DU/mm2
0.55
0.5
0.4
0.45
0.6
PKI55
- 87
DU/mm2
1.2
1.1
0.98
1.15
1.3
PKI55
- 6,5
DU/mm2
-
-
-
1.5

Figure 8

a)

REFINED ALIGNMENT

		Score	Identities	Positivities
+PKI55	MLYKLHDVCRQLWFSCPACHHRAMRICCPAQHHRTISVCKTI-LSSPPPLDSLPCM			
Macaca	MLYKLHDVCRQLRFSCPACHHWAMEICCPAQHYRTTSVCKAV-LSNPPPLDSSP--	102	83%	88%
Chr.10	MLEKLQAVSKWWWLSCPAHYHRALQLCCPARHH-AVSVCKAVILPSPPPLDSLPCM	80,5	64%	76%
Chr.15	MLYKMHAACKWLQFSCPA---WAMQICYPAQCHWAISVHKAVLLTSPPPQDPLPCM	64,7	60%	68%
Chr.20	LYKLHAFCKLLWFSHLVCHLWAMQLSCPAHRHWT--WCKAVLLPSSPPLDSLPGM	61,6	58%	65%

+ query sequence

b)

PAIRWISE BLAST(+) NUCLEOTIDE SEQUENCES

	Accession number	Start codon	Promoter	TATA box	Transcription factors	E value
cDNA clone (++)	AL137534	88535	-102	-127	404bp	
Macaca Fascicularis	AB050412	2524	-109	-129	404bp	0
Chr. 10	AL356865	65940	-101	-126	253bp	2*10-76
Chr. 15	AC009432	134013	-118	-142	265bp	2*10-46
Chr. 20	AL050403	16933	-121	-151	212bp	2*10-57

(+) TBLASTN 2.2.1
(++) query sequence

Figure 9

Comments for pRc/RSV
5224 nucleotides

RSV promoter: bases 209-605
Polylinker: bases 606-705
BGH Poly A signal: bases 706-932
F1 origin: bases 988-1510
SV40 promotor: bases 1572-1897
SV40 origin of replication: bases 1766-1851
Neomycin gene: bases 1903-2697
SV40 Poly A signal: bases 2701-2910
ColE1 origin: bases 3233-3756
Ampicillin resistance gene: bases 4240-5100
pUC backbone, origin and β-lactamase gene: begins at base 3009
The unique sites in the polylinker are: HindIII, Spe I, BstX I, Not I, and Xba I

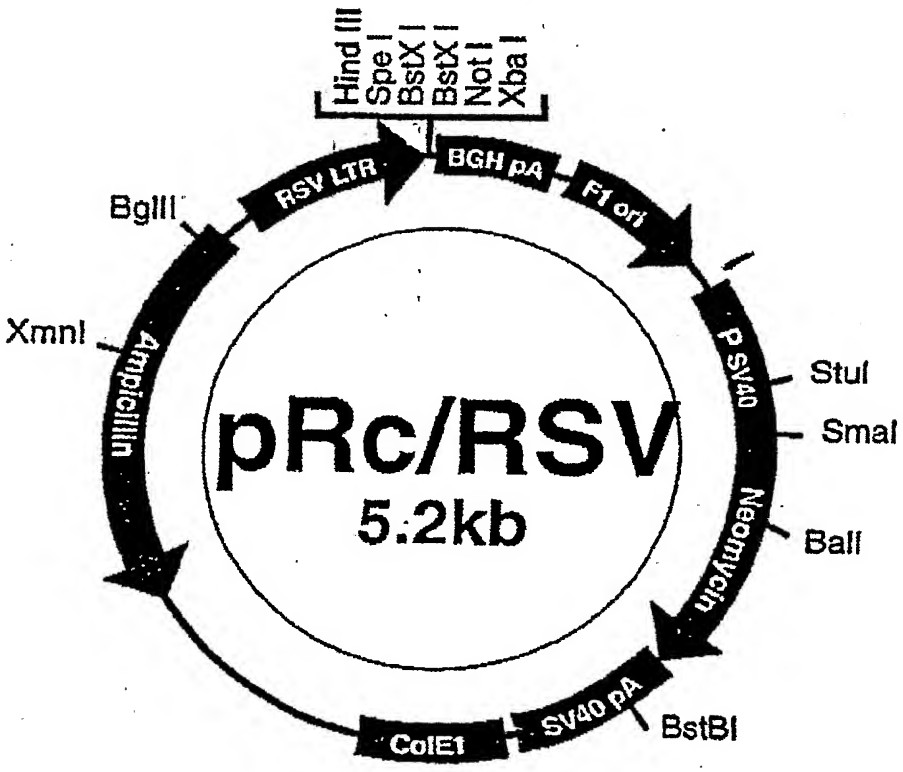


Figure 10

	0h	24h	48h	72h	96h	120h	144h	168h
Jurkat	100	137	300	462	800	1225	1733	1783
RSV	100	142	225	325	450	775	1258	1392
RSV-Senso	100	133	200	282	400	538	1100	1083
RSV-Anti	100	163	275	343	550	1012	1608	1700

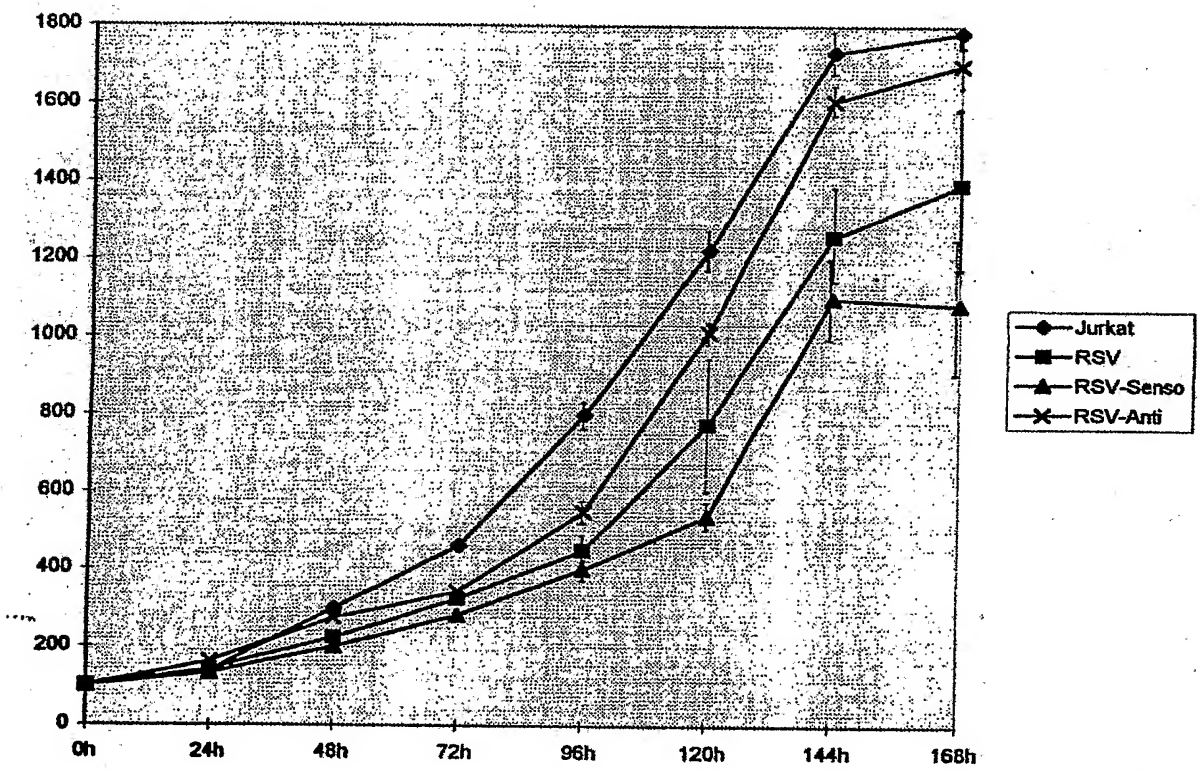


Figure 11

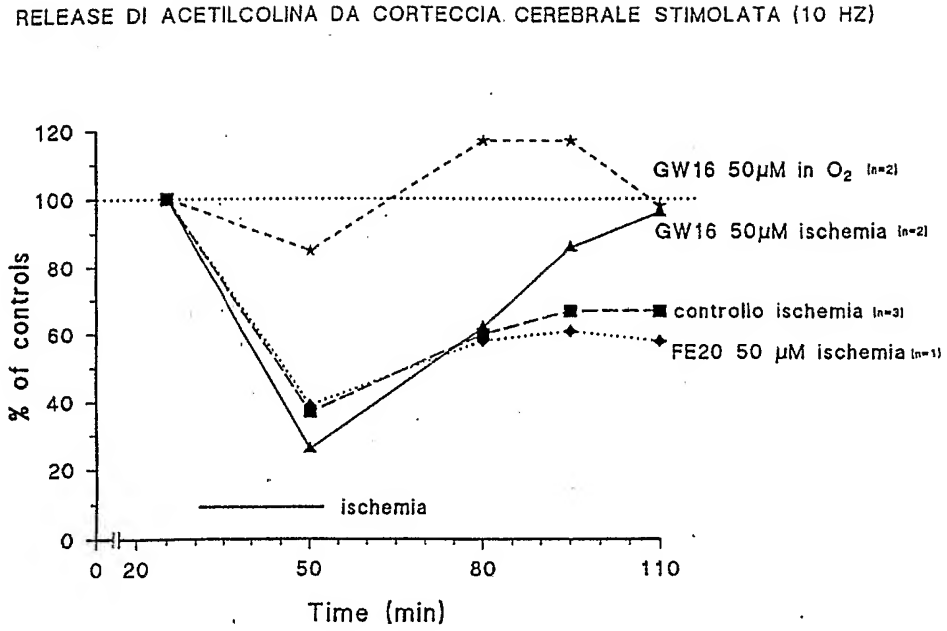

RELEASE DI ACETILCOLINA DA CORTECCIA CEREBRALE STIMOLATA (10 HZ)
% of controls
120
100
80
60
40
20
0
0 20 50 80 110
Time (min)
ischemia
GW16 50µM in O2 (n=2)
GW16 50µM ischemia (n=2)
controllo ischemia (n=3)
FE20 50 µM ischemia (n=1)

Figure 12

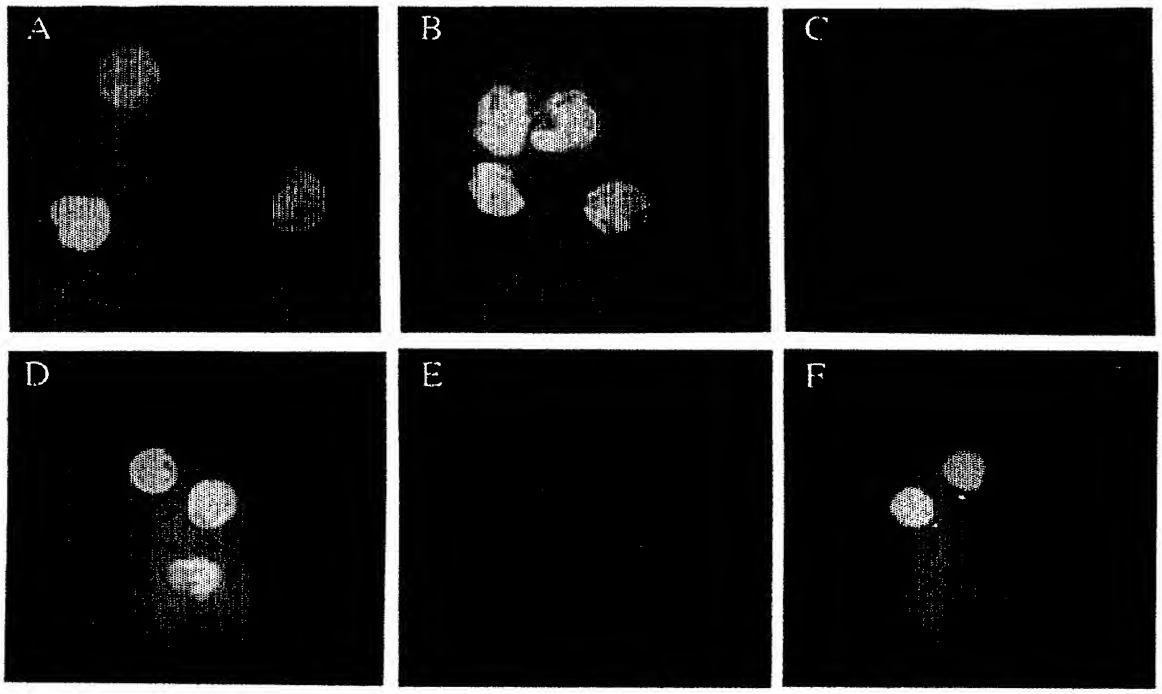
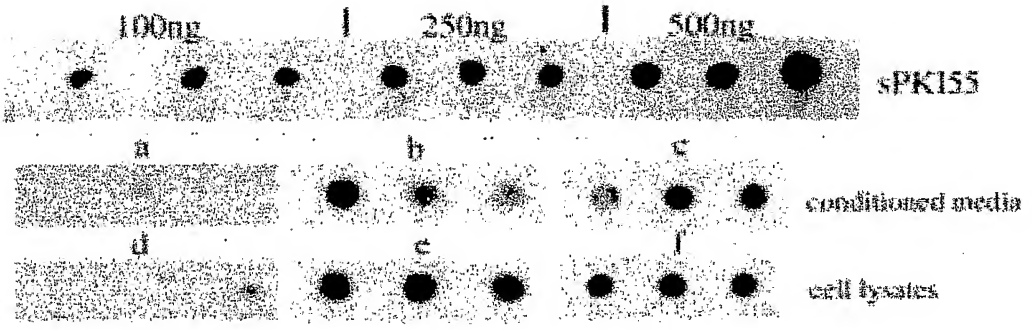


Figure 13


100ng
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500ng
sPK155
a
b
c
conditioned media
d
e
f
cell lysates

Figure 14

SEQUENCE LISTING

<110> Gandini, Enrico

<120> Protein Kinase C modulators, their aminoacid and nucleotide sequences and uses thereof

<130> 3099PT

<150> EP 02006861.5
<151> 2002-03-26

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Met Leu Tyr Lys Leu His Asp Val Cys Arg Gln Leu Trp Phe
1 5 10

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Ser Cys Pro Ala Cys His His Arg Ala Met Arg Ile Cys Cys Pro Ala
15 20 25 30

caa cac cac agg acc att tct gta tgt aag aca att cta tcc agc ccg 1467
Gln His His Arg Thr Ile Ser Val Cys Lys Thr Ile Leu Ser Ser Pro
35 40 45

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Pro Pro Leu Asp Ser Leu Pro Cys Met
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20 25 30

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35 40 45

Leu Asp Ser Leu Pro Cys Met
50 55

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Met Leu Glu Lys Leu Gln Ala Val Ser Lys Trp Trp
1 5 10

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Trp Leu Ser Cys Pro Ala His Tyr His Arg Ala Leu Gln Leu Cys Cys
15 20 25

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Pro Ala Arg His His Ala Val Ser Val Cys Lys Ala Val Ile Leu
30 35 40

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1 5 10

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15 20 25

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Leu Trp Phe Ser His Leu Val Cys His Leu Trp Ala Met Gln Leu Ser
15 20 25

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Cys Pro Ala His Arg His Trp Thr Val Cys Lys Ala Val Leu Leu Pro
30 35 40

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/03121

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K5/103 C07K7/04 C07K16/18 C12N15/12
C12N5/10 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! EBI; 25 October 2000 (2000-10-25) OSADA ET AL: "Isolation of full-length cDNA clones from macaque brain cDNA libraries" retrieved from SRS Database accession no. AB050412 XP002224924 cited in the application * Identical with SEQ 10; further, overlaps 77% in the region 2-1524 (APPL/SEQ 1) : 1193-2717 * --- -/--	25,29

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2003	30/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Korsner, S-E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/03121

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL 'Online! EBI; 5 November 2001 (2001-11-05) ROSEN ET AL: "Polynucleotides encoding digestive system antigens..." Database accession no. AAK91238 XP002224794 * Corresponds to WO-A-0155314, published on 02.08.01 without sequences (CD-ROM); sequence 4814 (3904 bp) overlaps 100% in the region 1-599 (APPL/SEQ 1): 683-2281 * ---	1-71
A	DATABASE EMBL 'Online! EBI; 27 January 2000 (2000-01-27) OTTENWAELDER ET AL: "(Homo sapiens mRNA...)" retrieved from SRS Database accession no. AL137534 XP002224925 cited in the application * Overlaps >99% in the region 1-1531 (APPL/SEQ 1) : 1082-2612; 1 mismatch * ---	1-71
L	DATABASE EMBL 'Online! EBI; 24 May 2002 (2002-05-24) BIRSE ET AL: "Novel 1405 isolated polypeptides..." Database accession no. ABL90383 XP002224926 * Corresponds to WO-A-0190304, published on 29.11.01 without sequences (CD-ROM); sequence 945 (2290 bp) overlaps >99% in the region 1-1599 (APPL/SEQ 1) : 173-1772; a total of 3 gaps * -----	1-71

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/03121

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7-10 (all partly) and dependent claism, as applicable
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 7-10 (all partly) and dependent claism, as applicable

Present Claims 7-10 relate to an extremely large number of possible peptides, because at least 4 amino acids from "SEQ.ID.NO. 2" would be:

Firstly: (a) 52 tetramers + (b) 51 pentamers etc, of the original SEQ.ID.NO 2
AND
secondly, a large cascading number of peptides in view of the condition of Claim 1, namely the same calculations for every alternative SEQ.ID.NO within the condition of Claim 1 (55% identity)
AND
thirdly, further variations in view of Claims 8-10, namely optional substitutions, deletions or insertions.

Note also that Claim 11 is unclear as to whether N-terminal region means the first four amino acids, and that the term comprising is non-limiting.

Consequently, the search has been restricted to at least 4 amino acids from the first ten amino acids of original SEQ.ID.NO. 2.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.